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(54) Title: SERINE PEPTIDASE MODULATORS

(57) Abstract

The present invention relates to new compounds having modulatory (inhibitory and stimulatory) activity on serine peptidases and proteases in general and dipeptidyl peptidase IV, prolyl oligopeptidase (PO), dipeptidyl peptidase II (DPP II), fibroblast activation protein α (FAPα), lysosomal Pro–X carboxypeptidase and elastase in particular. These new compounds can be used for the treatment of a variety of disease states in which these peptidases are involved.

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SERINE PEPTIDASE MODULATORS

Field of the invention

The present invention relates to novel modulators (inhibitors and stimulators) of serine peptidases and proteases in general and dipeptidyl peptidase IV, prolyl oligopeptidase (PO), dipeptidyl peptidase II (DPP II), fibroblast activation protein α (FAPα), lysosomal Pro-X carboxypeptidase and elastase in particular. The invention further relates to the preparation and use of these compounds for selective modulation (inhibition or stimulation) of serine peptidases and proteases and to pharmaceutical preparations comprising them. The terms "peptidase" and "protease" are used interchangeably.

Background of the invention

Serine peptidases/proteases, like granzymes, mast cell tryptase, elastases, trypsin-like enzymes, prolyl oligopeptidase, dipeptidyl peptidase II and dipeptidyl peptidase IV are involved in various processes that take place in the body, such as blood coagulation, inflammation, immune response, and control of peptide hormone metabolism in general. Although serine peptidases are a physiological necessity they may also constitute a potential health hazard in case serine peptidase activity in the body is not controlled.

Serine peptidases have been described to be involved in various medical indications. Blood coagulation serine proteases are for example responsible for vascular clotting as well as cerebral and coronary infarction. Chymotrypsin-like enzymes and plasmin are involved in tumour invasion, tissue remodeling and clot dissociation. Pancreatitis, emphysema, rheumatoid arthritis, inflammation and adult respiratory distress syndrome may in some instances be caused by the uncontrolled proteolysis by other serine proteases such as elastase.

Serine peptidases form a larg group with many members that are divided into clans and families. One member of the clan SC is dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5), which is a highly specific exopeptidase with a serine type mechanism of protease activity, cleaving off dipeptides from the amino-terminus of peptides with proline or alanine at the penultimate position. In addition the slow release of dipeptides of the type X-Gly or X-Ser is reported for some naturally occurring peptides. DPP IV is constitutively expressed on epithelial and endothelial cells of a variety of different tissues, and is also found in body fluids. In the hematopoietic system, DPP IV was identified as the leukocyte antigen CD26.

Prolyl oligopeptidase (PO, EC 3.4.21.26) was discovered in the human uterus as an oxytocin-degrading enzyme. The enzyme shows a high specificity for proline residues and hydrolyses the peptide bond at its carboxyl side, provided the proline is not at the peptide amino-terminus. This endopeptidase has like DPPIV, a serine type mechanism and it is characterised by its activity on oligopeptides. PO cleaves specifically the Pro-Xaa bond in biological active peptides (substance P, ocytoxin, vasopressin, gonadoliberin, bradykinin, neurotensin) and it is likely to participate in the in vivo regulation of their actions. A role for PO in memory and other neural processes has been proposed (Yoshimoto T. and Ito K. in Handbook of proteolytic enzymes, eds. Barrett et al., Academic Press, 1998, p. 272-374).

Fibroblast activation protein α (FAPα) was discovered as a cell surface antigen of cultured normal fibroblasts. Its expression in vivo revealed to be very restricted on normal cells. In contrast, activated tumor stromal fibroblasts found in certain carcinomas express high levels of FAPα. The biological role of FAPα expression remains to be elucidated but speculations on functions in tissue remodeling and repair hav been made (Rettig, FAPα in Barrett supra, p. 385-389).

Dipeptidyl peptidase II (DPPII, EC 3.4.14.2) releases N-terminal dipeptides from oligopeptides, provided their N-termini are unsubstituted. The preferred P1 residues are Ala and Pro. An increase in serum DPPII has been observed in cancer patients and extremely high levels of DPPII are present in human carcinoma cells. DPPII can be inhibited by the classical (unspecific) inhibitors of serine type peptidases (J.K.McDonald in Barrett, supra, p. 408-411).

Elastases are defined by their ability to release soluble peptides from insoluble elastin fibers by a proteolytic process called elastinolysis. Elastase belongs to the chymotrypsin family of leucocyte serine-type proteases. Human leucocyte elastase (EC 3.4.21.37) preferentially cleaves peptides with a Val in P1 but also peptide bonds with Ala, Ser and Cys in P1 are hydrolyzed and it is believed to possess an extended substrate-binding site. The possible involvement of leucocyte elastase in inflammatory diseases, triggered the search for development of specific inhibitors. Moreover, a pathological role in lung emphysema, cystic fibrosis and adult respiratory distress syndrome has been suggested (J. Bieth in Barrett, supra, p. 54-60; D.Farley et al. in Pharmaceutical Enzymes, ed. A. Lauwers and S. Scharpé, Marcel Dekker, Inc., 1997, p. 306-326).

Lysosomal Pro-X carboxypeptidase (prolylcarboxypeptidase, angiotensinase C, EC 3.4.16.2) cleaves C-terminal amino acids from peptides with the general structure X-Pro-Y, where X is either a blocking group, another protected amino acid, or a peptide, and Y is an aromatic or aliphatic amino acid with a free carboxylic group. The enzyme is recovered from the lysosomal fraction of different tissues. Although the enzyme has an acidic pH optimum for small synthetic substrates (pH 5.0), it retains 50% of its maximal activity at physiological pH towards larger peptide substrates. (Des-Arg9)-bradykinin and angiotensin II are possible natural substrates for lysosomal Pro-X

carboxypeptidase (Tan, F. and Erdös, E. in Barrett et al., supra, p. 405-407)

Because of their role in various physiological processes it is desirable to interfere in the activity of serine peptidases. Such interference can be either stimulation or inhibition. Various types of serine peptidase inhibitors have been described in for example EP-764 151 of the present inventors. The application inter alia describes compounds of the general formula Z-Xaa-Y' wherein Z may or may not be present and is a protecting group, Xaa represents a dipeptide or an amino acid and Y' may be a phosphonate, such as a diphenyl phosphonate. Further research of the present inventors has revealed that the toxicity thereof is not yet acceptable and the potency/efficacy are not sufficient.

US-5,543,396 of Powers et al. relates to proline phosphonate derivatives. The phosphonate may be substituted with one or two phenyl groups which in turn may be mono-, di- or trisubstituted with a halogen, C_1-C_6 alkyl, C_1-C_6 perfluoralkyl, C_1-C_6 alkoxy, NO_2, CN, OH, CO_2H, amino, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino, C_1-C_6 acyl, and C_1-C_6 alkoxy-CO-, C_1-C_6 alkyl-S-. The present inventors, in the research that led to this invention, developed independently the same compounds. However, they found that regarding toxicity, stability and efficacy these compounds did not perform optimally.

It is therefore the object of the present invention to provide inhibitors of serine peptidases/ proteases that have a more optimal combination of inhibitor capacity, stability in plasma, safety, bioavailability, duration of action and straightforward synthesis. In addition, the invention has for its object to provide compounds that have a stimulating activity on serine peptidases/ proteases. These two types of compounds of the invention will also be identified herein as "modulating compounds". More in particular, the invention provides compounds having such a more optimal combination for modulating the activity of DPP IV, PO,

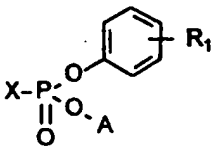
DPP II, FAPα, lysosomal Pr -X carboxypeptidase and elastas .

Thus, in the research that led to the pr sent invention, the influ nce of different functional groups on the inhibitory (or stimulatory) activity of phosphonates was investigated. Prolylpyrrolidine diphenyl phosphonates were synthesized, substituted on the phenyls with hydroxyl, methoxy, acylamino, sulfonylamino, ureyl, methoxycarbonyl and alkylaminocarbonyl groups. The phenylesters were also replaced by other groups with good leaving group capacities such as trichloroethyl and trifluoroethyl. The inhibitory activity in vitro and in vivo on DPP IV and other serine peptidases, the stability and specificity of these compounds was tested.

It was then found that compounds as claimed in claim 1 are very potent modulators, in particular inhibitors, of serine peptidases/proteases in general and DPP IV, DPP II, PO, FAPα, lysosomal Pro-X carboxy-peptidase and elastase in particular. The compounds as listed in claim 12 were found to be potent inhibitors of DPP IV and PO.

The compounds of the invention are based on peptides. These peptides are constituted by either naturally occurring amino acids or other amino acids. The C-terminal carboxyl function is replaced by a phosphonate group.

The compounds of the invention are represented by the general formula I:



(I)

wherein

- A is ---(R2) or H or C_1-C_6 alkyl or halogenoalkyl, except perfluoroalkyl,

- the phenyl group is mono-, di- or trisubstituted with R1 or R2;
- X is a peptide- or amino acid-derived moi ty;
- A and th phenyl group substituted with R1 may optionally form a biphenyl diester;
- all R1 substituents and R2 substituents are each independently selected from the group consisting of:

a) C_1-C_6 acylamino;

b) aroylamino, optionally substituted at the o- and/or p- and/or m- position with alkyl, in particular C_1-C_6 alkyl, and/or a halogen;

c) C_1-C_6 alkylsufonylamino;

d) arylsulfonylamino, optionally substituted at the o- and/or p- and/or m- position with alkyl, in particular C_1-C_6 alkyl, and/or a halogen;

e) α aminoacylamino wherein the α aminoacyl represents a side chain blocked or unblocked α-amino acid residue with the L, D or DL configuration at the α-carbon atom selected from the group consisting of:

alanine, methionine, methionine sulfoxide, arginine, homoarginine, phenylalanine, aspartic acid, proline, hydroxyproline, asparagine, serine, cysteine, threonine, histidine, glycine, tyrosine, glutamic acid, pyroglutamic acid, tryptophan, glutamine, valine, norvaline, isoleucine, lysine, leucine, norleucine, thioproline, homoproline, 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid (Tic), 2,3-dihydroindol-2-carboxylic acid, α-naphtylglycine, α-phenylglycine, 4-amidinophenylglycine, 4-phenylproline, 4-amidinophenylalanine, O-benzyl tyrosine, omega-acetyl lysine, α-aminobutyric acid, citrulline, homocitrulline, ornithine, o-methylserine, o-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, homoserine, 4-dehydroproline, penicillamine, β-(2-thienyl)alanine, NH_2-$CH(CH_2CHEt_2)$-COOH, α-

aminoheptanoic acid, NH_2-CH(CH_2-1-naphthyl)-COOH, NH_2-CH(CH_2-2-naphthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH[CH-(cyclohexyl)$_2$]-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH[CH-(cyclopentyl)$_2$]-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH[CH-(cyclobutyl)$_2$]-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, NH_2-CH[CH-(cyclopropyl)$_2$]-COOH, 5,5,5-trifluoroleucine, hexafluoroleucine, (S)-azetidine-2-carboxylic acid, (S)-pipecolic acid, (S)-oxazolidine-4-carboxylic acid, (R)-thiazolidine-4-carboxylacid (L-thioproline), sarcosine;

f) residue selected from the group consisting of 3-aminobenzoic acid, ε-aminocaproic acid, β- alanine;

g) Y-NH-CO-NH-;

h) Y'O_2CCH(NHCO-Y)-CH_2-;

i) Y'NHCO-;

j) CH_3-O-CO-Y'-NH-CO-;

k) CH_3-CH_2-O-CO-Y'-NH-CO-;

wherein Y is C_1-C_6 alkyl, aryl or H and Y' is C_1-C_6 alkyl,

and pharmaceutically acceptable salts thereof.

In a specific embodiment of the invention X is a moiety of the general formula $(AA)_p$-aa-,

wherein:

p indicates that there may be 0, 1, 2, 3, 4 or 5 residues AA, which can be the same or different within one molecule;

AA and aa are selected from one of the following:

a) α-amino carboxylic acids with in α position an optionally substituted C_1-C_6 alkyl or aryl or aralkylmoiety;

b) alanine, methionine, methionine sulfoxide, arginine, homoarginine, phenylalanine, aspartic acid, proline, hydroxyproline, asparagine, serine, cysteine, threonine, histidine,

glycine, tyr sine, glutamic acid, pyroglutamic acid, tryptophan, glutamine, valine, norvaline, isoleucine, lysin , leucin , norleucine, thioproline, homoproline, 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid (Tic), 2,3-dihydroindol-2-carboxylic acid, α-naphtylglycine, α-phenylglycine, 4-amidinophenylglycine, 4-phenylproline, 4-amidinophenylalanine, O-benzyl tyrosine, omega-acetyl lysine, α-aminobutyric acid, citrulline, homocitrulline, ornithine, o-methylserine, o-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, homoserine, 4-dehydroproline, penicillamine, β-(2-thienyl)alanine, NH_2-CH(CH_2CHEt_2)-COOH, α-aminoheptanoic acid, NH_2-CH(CH_2-1-naphthyl)-COOH, NH_2-CH(CH_2-2-naphthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH[CH-(cyclohexyl)$_2$]-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH[CH-(cyclopentyl)$_2$]-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH[CH-(cyclobutyl)$_2$]-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, NH_2-CH[CH-(cyclopropyl)$_2$]-COOH, 5,5,5-trifluoroleucine, hexafluoroleucine, (S)-azetidine-2-carboxylic acid, (S)-pipecolic acid, (S)-oxazolidine-4-carboxylic acid, (R)-thiazolidine-4-carboxylacid (L-thioproline), 3-aminobenzoic acid, sarcosine, ϵ-aminocaproic acid, β-alanine,

wherein the alpha amino residue may be side chain blocked or unblocked and has the L, D, or DL configuration at the alpha carbon atom;

and pharmaceutically acceptable salts thereof.

In an alternative embodiment of the invention X is M-$(AA)_p$-aa-

wherein:

p, AA and aa are as defined above; and

M is selected from:

a) the group consisting of optionally substituted $-CONH_2$, $-CSNH_2$, $-SO_2NH_2$, phenyl-SO_2-, phenyl-CH_2SO_2-, 2-furyl-acryloyl; and

b) the group of protecting groups consisting of: acetyl, adamantyloxycarbonyl, benzyl-oxycarbonyl, benzoyl, benzyl, t-butoxycarbonyl, t-butyl, 2,4-dinitrophenyl, formyl, fluorenylmethoxycarbonyl, 4-methoxybenzyl, tosyl, trifluoro-acetyl, trityl, phthaloyl, phenylalkylcarbonyl, 2-indanylacetyl, 2-(1,2,3,4-tetrahydronaphtyl)acetyl, 4-(4-benzylphenoxy)alkyl;

In a specific embodiment X represents AA-aa-, wherein aa is proline and AA is as defined above. In an alternative embodiment X represents AA-aa-, wherein AA and aa are both proline. In case aa is alanine, R1 and R2 may further be selected from:

l) H, halogen, NO_2, CN, OH, COOH

m) amino, C_1-C_6 alkylamino, C2-$C_1$2 dialkylamino,

n) C_1-C_6 acyl

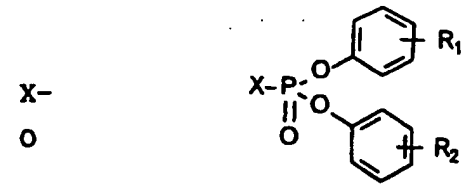
o) C_1-C_6 alkoxy-CO-

p) C_1-C_6 alkyl-S-.

In such compounds aa is alanine and preferably at least the AA coupled to aa is proline or phenylalanine. Preferred examples of the compounds are Phe-Ala-diphenyl-phosphonate or Pro-Ala-diphenylphosphonate and pharmaceutically acceptable salts thereof.

The invention can be divided in three groups of compounds.

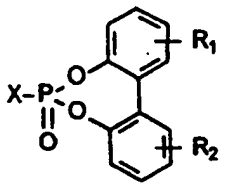
In preferred compounds of this invention the phosphonate group is a diphenyl-phosphonate group (indicated with the symbol P(OPh)2), which is preferably substituted. The first group thus consists of compounds of the general formula II:



(II)

wherein the substituents R1, R2 and X are as defined above (group 1).

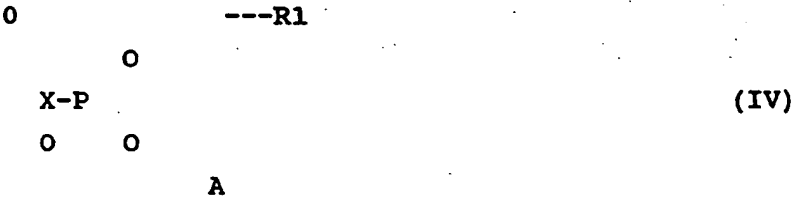
Alternatively, the compounds are 2,2' biphenyl diesters of α-aminoalkyl phosphonic acid having the general formula III:



(III)

wherein the substituents R1, R2 and X are as defined above (group 2).

The third group (group 3) consists of compounds having the general formula IV:



(IV)

wherein X and R1 are as defined above, A is H or C_1-C_6 alkyl or halogenoalkyl, except perfluoroalkyl.

Specific members of these three groups will be listed below.

For inhibition of the serine protease cathepsin G, X is preferably selected from Cbz-Gly-Leu-Phe-, Z-Phe-Pro-Phe, and Suc-Val-Pro-Phe-. For prolyl oligopeptidase X may be selected from among the following: Cbz-Gly-Gly-Pro-, Cbz-Pro-Pro-, Boc-Val-Pro-Val-, MeO-Suc-Ala-Ala-Ala-Val-, MeO-Suc-Ala-Ala-Pro-Val-. For DPP IV, X is as follows: Ala-Pro-, Pro-Pro-, Ala-Pip-, Phe-Pro-, Ile-Pro-, Arg-Pro-, pF-Phe-Pro-, cyclohexylala-Pro-, Pro-azetidine-, Phe-azetidine-, Lys-Pro-, Lys-azetidine-. X is selected from among Suc-Lys(Cbz)-Val-Pro-Val-, Z-Ala-

Ala-Ala and Boc-Val-Pro-Val- in case the enzyme to be inhibited is human leukocyte elastase. For Granzyme A, X may be selected from Cbz-(4-amidinophenylalanine)-, Z-Met-, 3-phenyl propanoyl-Pro-(4-aminophenylalanin)-, Cbz-Thr-(4-amidinophenylglycine)-, and Boc-D-Phe-Pro-(4-amidinophenylalanine)-. X may be Z-Phe-Pro-Phe-, Z-Phe-, Suc-Val-Pro-Phe-, MeO-Suc-Ala-Ala-Pro-Phe-, or MeO-Suc-Ala-Ala-Ala-Phe- when the enzyme to be inhibited is chymotrypsine. For trypsin-like serine-type proteases, X may be selected from among Cbz-Orn-, Cbz-Lys-Ala-, Cbz-Lys, Cbz-HomoLys-, Cbz-(4-amidinophenylalanine)-, Cbz-(4-amidinophenylglycine)-, Ph-CH_2-SO_2-Gly-Pro-(4-amidinophenylglycine)-, 3-(2-furyl)acryloyl-(4-amidinophenylglycine), Cbz-Lys-(4-amidinophenylglycine)-, Cbz-Lys-Ala-(4-amidinophenylglycine)-, Cbz-Thr-(4-amidinophenylglycine)-, 3-(2-furyl)acryloyl-(4-amidinophenylalanine)-, Cbz-Ala-(4-amidinophenylglycine)-, Cbz-Ala-Ala-Ala-(4-amidinophenylglycine)-, 2-phenoxybenzoyl-Pro-(4-amidinophenylglycine)-, 3-phenoxybenzoyl-Pro-(4-amidinophenylglycine)-, 3-phenyl propanoyl-Pro-(4-amidinophenylalanine)-, 3,3-diphenyl propanoyl-Pro-(4-amidinophenylglycine)-. For inhibition of V8 protease of S.aureus X can be either Acetyl.Glu- or Acetyl.Asp-.

R1 and R2 are preferably selected from the group consisting of: 3-AcNH, 4-AcNH, 4-$MeSO_2NH$, 3-H_2NCONH, 3-H_2NCONH, 4-(N-Bz-Gly-NH), 4-(H-Gly-NH), 4(H-(S)-Ala-NH), 4-((S)-Pyr-NH), 4-((2S)-$MeO_2CCH(NHAc)CH_2$), 4-MeO_2C, 4-(EtO_2CCH_2NHCO), 4-($MeO_2C(CH_2)_2NHCO$), 4-$CH_3(CH_2)_2NHCO$.

Particularly preferred compounds of group 1 (formula II) are the following:

- Di(3-acetamidophenyl) 1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**10d**);
- Di(4-acetamidophenyl) 1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**10e**);
- Di(4-methylsulfonylaminophenyl) 1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**10f**);

- Di(3-ureylphenyl) 1-(benzyl xycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**10g**);
- Di[4-(N-benzoylglycylamino)phenyl]-1-(benzyl-oxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phospho-nate (**10h**);
- Di[4-(N-benzyloxycarbonylglycylamino)phenyl]-1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**10i**);
- Di[4-(N-benzyloxycarbonyl-(S)-alanylamino)phenyl]-1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**10j**);
- Di[4-((S)-pyroglutamylamino)phenyl]-1-(benzyloxy-carbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**10k**);
- Di{4-[-(S)-(2-methoxycarbonyl-2-acetamido)ethyl]-phenyl}1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**10l**);
- Di(4-methoxycarbonylphenyl)1-(tert-butyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**10m**);
- Di{4-[(ethoxycarbonyl)methylaminocarbonyl]phenyl} 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (**10n**);
- Di{4-[2-(methoxycarbonyl)ethylaminocarbonyl]phenyl} 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (**10o**);
- Di[4-(n-propylaminocarbonyl)phenyl] 1-(benzyloxy-carbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (**10p**);
- Di(3-acetamidophenyl) 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (**11d**);
- Di(4-acetamidophenyl) 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (**11e**);
- Di(4-methylsulfonylaminophenyl) 1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11f**);
- Di(3-ureylphenyl) 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (**11g**);
- Di[4-(N-benzoylglycylamino)phenyl]-1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11h**);

- Di[4-(N-glycylamino)phenyl]-1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate trihydrochlorid (**11i**);
- Di(4-(S)-alanylaminophenyl)-1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate trihydrochloride (**11j**);
- Di(4-(S)-pyroglutamylaminophenyl)-1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11k**);
- Di{4-[-(S)-(2-methoxycarbonyl-2-acetamido)ethyl]-phenyl} 1-((S)-prolyl)pyrrolidine-2-phosphonate hydrochloride (**11l**);
- Di{4-[(ethoxycarbonyl)methylaminocarbonyl]phenyl} 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**11n**);
- Di{4-[2-(methoxycarbonyl)ethylaminocarbonyl]phenyl) 1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11o**);
- Di[4-(n-propylaminocarbonyl)phenyl] 1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11p**).

A compound having an inhibitory activity for DPP IV, is for example 2,2'-Biphenyl 1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**19**) or a pharmaceutically acceptable salt.

Other specific compounds of group 2 (formula III) are the following:

- 2,2'-Biphenyl 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (**17a**);
- 2,2'-Biphenyl 1-(t-butyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (**17b**).

An example of a compound of group 3 (formula IV) is:

- 2-(2'-Hydroxyphenyl)phenyl methyl 1-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**18**);

The advantage of compounds of the invention over Powers et al. (supra) follows from the comparison of the properties of the leaving groups that are created from Powers' compounds and from the compounds of the invention when the inhibitors enter into a covalent binding with the serine in the active center of the

peptidases. The information c mes from the Merck Index, 12th edition, Merck & Co, Inc. USA (1996).

Powers' leaving groups are am ng thers 2-methylphenol (o-cresol), 3-methylphenol (m-cresol) and 4-methylphenol (p-cresol). The compounds belong to the group of cresols, which are desinfectants. These compounds are toxic to humans. Chronic poisoning from oral or percutaneous absorption may produce digestive disturbances, nervous disorders, vertigo, skin eruptions, jaundice, oliguria, uremia. Another leaving group in Powers' compounds is 4-hydroxybenzoic acid methylester (methyl paraben, Nipagin M) which is also a preservative in foods beverages and cosmetics and of which allergic reactions are frequently observed. 1,4-Benzenediol (hydroquinone) is a photographic developer and reducer and is used as an antioxidant. At very low concentrations there is no systemic toxicity. However, ingestion of more than 1 g results in nausea, vomiting, shortness of breath, cyanosis, convulsions and collapse. It is lethal in a dose above 5 g.

The leaving groups of the compounds of the invention however, are for example 4-hydroxyacetanilide (paracetamol, compound **11e**) which is used commonly as a very safe analgesic/antipyretic drug. Another leaving group is 4-hydroxyhippuric acid ethyl ester (compound **11n**) of which no toxicity has been noticed, and hippuric acid is known to be a metabolite occurring in human metabolism and a normal constituent in human urine.

From the above it follows that in comparison to Powers' compounds, the compounds of the invention do not present safety problems which could obstruct their further pharmaceutical development.

The compounds of the invention can be used for the therapy of pathological states associated with excessive, impaired or unbalanced activity of said enzymes.

Starting from the available information on the corr lation between particular serine protease activities

and various disease states the skilled person will be able to define therapeutical utilities for the modulatory compounds of th invention. Hereinbelow examples of such disease states will be listed and supp rt for th ir utility given.

Thus, the invention relates to the compounds for use as a therapeutical agent. In particular, the invention relates to the compounds for use in the treatment or prophylaxis of inflammation, vascular diseases, organ specific or systemic auto-immune diseases (e.g. Graves' disease or multiple slerosis, inflammatory bowel disease), joint diseases, muscle diseases, neurological diseases, obesity, diseases associated with benign and malign cell transformation, spreading of malignant cells, conditions of glucose-intolerance, abnormal growth or growth retardation, rejection of foreign cells or tissues after transplantation, abnormalities in blood cell development, abnormal blood clotting, pain, or diseases of the central nervous system.

Support for the pharmaceutical utility of the compounds of the invention in the above indications follows from the following publications, which demonstrate the correlation of specific serine peptidases with medical indications. Inhibition of these enzymes can thus be used as treatment or prophylaxis.

The involvement of plasma prekallikrein or kallikrein in shock is described by W. Colman in Barrett, A., et al., supra, page 147-153.

The compounds of the invention can be used for prevention and treatment of thrombosis and conjunction therapy of acute myocardial infarction by specifically inhibiting Factor X (Vlasuk, G.P. in New Therapeutic Agents in Thrombosis and Thrombolysis, Sasahara, A., Loscalzo, J., eds (1997), pages 261-283), thrombin (Shafer, J.A. in New Therapeutic Agents in Thrombosis and Thrombolysis, (supra) pages 143-157) and Factor VII (factor VII-Tissue Factor) (Shafer, J.A. in New

Therapeutic Agents in Thr mbosis and Thrombolysis, (supra) pages 225-260).

Various serine peptidases are known to b involved in inflammation. That this is the case is described for elastases (Bank U. et al., in Cellular Peptidases in Immune Function and Diseases, Ansorge, S. & Langner, J., Plenum Press (1997), pages 231-242, D. Farley et al. in Lauwers, A. and Scharpé, S. eds., supra), kallikrein (Chao, J. in Barrett et al., supra, pages 97-101; Naidoo, Y. & Bhoola, K. in The kinin System, Framer, S.G., Academic Press (1998), pages 187-197); and Erdös, E.G. & Skidgel, R.A. in The Kinin System, supra, pages 112-141), cathepsin G (Flad, H.D. et al., in Cellular Peptidases in Immune Function and Diseases, supra, pages 223-230), DPP IV (Tanaka, S. et al., Immunopharmacology 40 (1998), 21-26), and granzymes (Berthou, C. et al., Pathol. Biol. 46 (1998), 617-624).

DPP IV (Cheng, H.C. et al. J. Biol. Chem. 273 (1998) 24207-24215), PO (Goossens et al., Eur. J. Clin. Chem. Clin. Biochem. 34 (1996), 17-22; and Ishino, T. et al., J. Biochem. 123 (1998), 540-545) and urokinase are involved in tumorigenesis and metastasis.

Holst, J.J. & Deacon, C. have described the potential of DPP IV inhibition in Type 2 diabetes or glucose intolerance (Diabetes 47 (1998), 1663-1670).

Elastase plays a role in autoimmunity diseases such as rheumatoid arthritis (Momohara, S. et al., Clin. Rheumatol. 16 (1997), 133-140; and Shinguh, Y. et al., Eur. J. Pharmacol. 337 (1997), 63-71; Barrett, supra, Lauwers & Scharpé, supra).

Korom, S. et al., (Transplantation 63 (1997), 1495-1500 have found an involvement of DPP IV in transplant rejection. Likewise, DPP IV has been found to be involved in growth abnormalities (Bai, P. & Chang, L.L., J. Pharm. Pharmacol. 47 (1995), 698-701; and Martin, R. et al., Biochim. Biophys. Acta 1164 (1993), 252-260) and hypertension and pre-eclampsia (Neudeck, H. et al., J. Reproductive Immunology 37 (1997), 449-458).

The latter two indications are also c rrelated with PO (Chappell, M.C. t al., Braz. J. Med. Biol. Res. 31 (1998), 1205-1212; and Umemura, K. et al., Br. J. Clin. Pharmacol. 43 (1997), 613-618).

PO is furthermore involved in muscle dystrophy (Kar, N. & Pearson, C., Clin. Chim. Acta 111 (1981), 271-273) and behavioral and neurochemical diseases (Toide, K. et al., Reviews in Neurosciences 9 (1998), 17-29).

DPP IV is a very versatile enzyme according to its localisation and different substrates and correlated with pain (Shane, R. et al., Brain Res. 815 (1999), 278-286) and obesity (Pederson, R. et al., Diabetes 47 (1998) 1253; Flint, A. et al., J. Clin. Investigation 101 (1998), 515-520) and tissue repair (Drucker, D. et al., Am. J. Physiol. 276 (1999), G79-91).

The present invention also includes derivatives which have been modified in the N-terminal amino acid side chain without abolishing the reactivity with the active site. Examples of such modifications are the incorporation of a radioactive label such as Iodine^{125} into tyrosine, extension of the side chain to attach biotin or a fluorophore. To improve the half-life in the circulation the peptide bond between the two amino acids may be replaced by a non-hydrolyzable bond. The incorporation of a radioactive label is useful in diagnostic methods using the modulating compounds.

The compounds are for example labeled for use in diagnostic and research methods such as fluorescence and radio-assays, imaging, <u>in situ</u> histochemical and cytochemical staining etc. as will be further explained hereinbelow.

According to a further aspect thereof, the invention relates to the use of the compounds for the preparation of a therapeutical composition for modulating (inhibiting or stimulating) the activity of serine proteases. Such therapeutical composition is then specifically intended for treatment and prophylaxis of the conditions listed above.

Furthermore, the invention provid s a pharmaceutical pr paration comprising one or more compounds of the invention and a suitabl excipient, carrier or diluent. Such pharmaceutical preparations are intended for the treatment and prophylaxis of the above conditions.

Acceptable excipients, carriers and diluents are well known and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). Preservatives, stabilizers, dyes and flavoring agents may be provided in the pharmaceutical compositions. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. In addition antioxidants and suspending agents may be used.

The compounds of this invention may be formulated and used as tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions or suspensions for injectable administration, aerosols, galenic preparations for topical and bucal administration and aerosols for nasal administration. If desired, absorption enhancing preparations (e.g. liposomes) or other appropriate delivery systems may be used. The amount of the active substance(s) in a dosage unit may vary between 0.001 mg and 1 g.

The compounds or pharmaceutical compositions of this invention can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. Such other therapeutic or diagnostic agent(s) can have a different dosage form or can be present in the same dosage form as the compounds of the invention. The dosage for the compounds of the present invention can range broadly depending upon the desired effects and the therapeutic indication.

The invention also relates to a method for <u>in vitro</u> inhibition or stimulation (modulation) of protease activity by means of a suitable concentration of a

compound of the invention. Such method is in particular useful wh n a protease inactivates a p ptide prior to measurement thereof in a peptide assay. The comp unds f the inventi n can be used t inhibit the degradation of the peptide substrate by the enzyme in such assay.

The compounds of the invention are also useful in a method for the 'ex vivo' inhibition of protease activity, such as the treatment outside the body of cells and organs for transplantation in order to avoid rejection thereof by the recipient body.

Furthermore, the compounds of the invention can be used in a method for in vivo inhibiting or stimulating (modulating) protease activity by means of administering to a living organism a suitable amount of a compound of the invention.

Such modulation (inhibition or stimulation) can be used for pharmacotherapy of disease states related to one of the following conditions: inflammation, organ specific or systemic auto-immune diseases, non-malignant disorder of leukocytes and/or immunoglobulins, rejection of cells or tissues after transplantation, tissue destructive and bone degenerative diseases, neuroendocrine dysfunction, glucose-intolerance, obesitas, functional gastrointestinal disorder, abnormal growth or growth retardation, thrombosis and hemorrhage, vascular and cardiopulmonary diseases, neurodegenerative and affective disorders, pain, diseases associated with neoplasia.

As already indicated above, the invention also relates to the diagnostic use of the compounds. Labeled inhibitors or stimulators can be used essentially in the same type of applications as labeled monoclonal antibodies, e.g. fluorescence and radioassays, cytofluorimetry, fluorescence activated cell sorting etc. The principles of such techniques can be found in immunochemistry handbooks, for example: Coligan, J. et al., Current Protocols in Immunology, vol. 1, 2 & 3, Wiley, 1998.

The compounds of the pr sent invention can also be used as affinity ligands f r analytical and preparative purpos s.

In cytochemistry and histochemistry labeled modulators can be used to directly visualize the cellular distribution of the target protease. The label can be fluorescent for fluorescence microscopy, radioactive for autoradiography, or electron dense for electron microscopy. The target structures can be whole cells, cells fixed onto slides or sections through solid tissue. A useful modification of these techniques is to use an indirect ("sandwich") assay employing the specific high affinity interaction between biotin and avidin (Coligan et al., supra).

For imaging or therapeutic targeting of tumours expressing high amounts of the target protease, modulators labeled with a suitable isotope can be injected. Eventually, after clearing of the excess modulator from the circulation, the tumour can be visualized by radioscintigraphy. The principles of imaging are summarized by A. Bamias & A.A.Epenetos (1995), Monoclonal antibodies, production, engineering and clinical application (M.A. Ritter and M.M. Ladyman, eds.) Cambridge University Press, Cambridge, pp. 222-246).

The modulators of the invention are suitable for the diagnostic applications described above, such as imaging and histochemical staining of the respective proteases end peptidases, because they form covalent, long-lived adducts with the proteases and peptidases. Because of their small size they are expected to penetrate tissue more easily than, for example, antibodies. In the case of DPP IV it was found that the modulators only recognize DPP IV which is native and enzymatically active. Formulations of the compounds to be used in diagnostic applications are also part of this invention.

Localisation of DPP IV by means of monoclonal antibody and by enzymatic activity is diagnostically useful and applicable to clinical material in the case of thyroid follicular tumours and thyroid papillary carcinoma (Kotani, T. et al., Int. J. Exp. Path. 73, 215-222 (1992) ; Kotani, T. et al., J. Path. 168, 41-45 (1992). In an analogous manner the compounds of the invention which form a stable adduct with DPP IV or other serine proteases may be used as a tool for diagnosing of certain disease states or monitoring the progression thereof or of treatments (Kurktschiev, D. et al., Clin. Exp. Immunol. (1999) 115, 144-146).

The invention is not limited to the compounds as claimed but also relates to pharmaceutically acceptable salts thereof.

The compounds of the invention may be pure diastereo-isomers or racemic mixtures.

Hereinbelow the invention will be described in more detail. The Examples disclose the synthesis of the modulating compounds of the invention, the inhibitory or stimulatory (modulatory) activity of the compounds of the invention on the catalytic activity of human DPP IV, PO, DPP II and elastase.

Serine protease activity can interfere with enzymatic assays for other substrates by cleaving the substrate used in the test and thereby giving either false positive (when a chromogenic substrate is cleaved) or false negative results (when a peptide substrate is degraded). The inhibitors of this invention can be used to inactivate contaminating serine proteases or peptidases before carrying on with the analysis.

This application relates to compounds that can either inhibit or stimulate the activity of serine proteases and peptidases. Because the activity of individual compounds of the group of compounds may be opposed (i.e. either inhibiting or stimulating) the general terms "modulating" and "modulation" are intended when reference is made to a group of compounds.

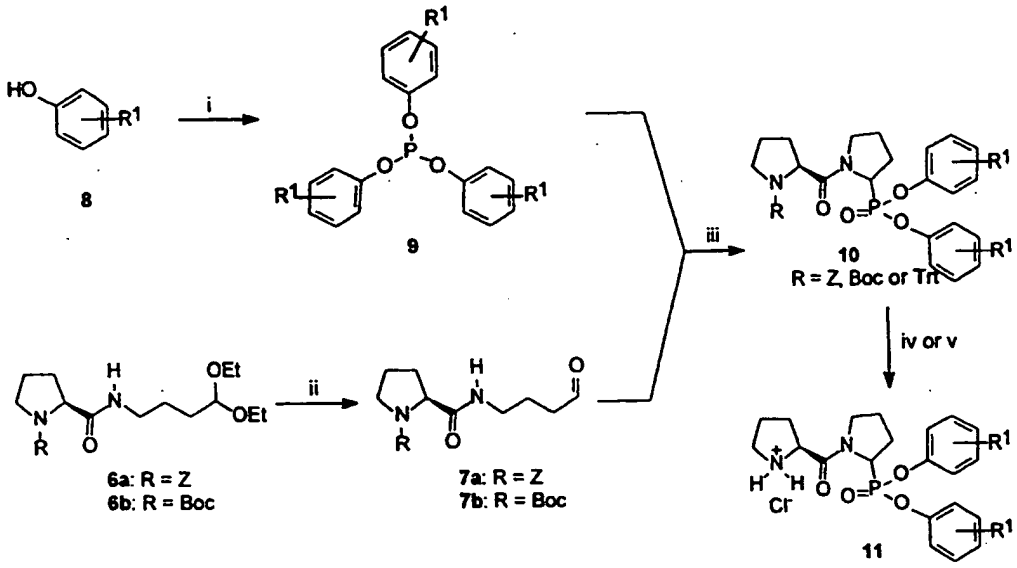
Individual compounds will have either a inhibitory or a stimulatory activity on individual serine p ptidases and proteases. A prerequisite of the invention is that the compounds that are claimed are active, i.e. have a modulatory (either inhibitory or stimulatory) activity on serine peptidases or serine proteases.

The terms "modulator", "inhibitor", "stimulator", "compound", "derivative" and "modulating compound" are used interchangeably.

The present invention will be further elucidated with reference to the following examples which are only given for illustration purposes and are in no way intended to limit the invention. The examples show how the previously reported diphenyl 1-(S)-prolyl-pyrrolidine-2-(R,S)- phosphonate (5) was used as a lead compound for the development of potent and irreversible inhibitors of dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5). The synthesis of a series of diaryl 1-(S)-prolylpyrrolidine- 2-(R,S)-phosphonates with different substituents on the aryl rings started from the corresponding phosphites. A good correlation was found between the electronic properties of the substituent and the inhibitory activity and stability. The most striking divergence of this correlation was the high potency combined with a high stability of the 4-acetylamino substituted derivative (11e). For this compound no cytotoxicity in human peripheral blood mononuclear cells could be observed and it also has favourable properties in vivo. Therefore di(4-acetamidophenyl) 1-(S)-prolyl-pyrrolidine-2-(R,S)- phosphonate (11e) is considered as a major improvement and will be a highly valuable DPP IV inhibitor for further studies on the biological function of the enzyme and the therapeutic value of its inhibition.

In the Examples reference is made to the following schemes and figures:

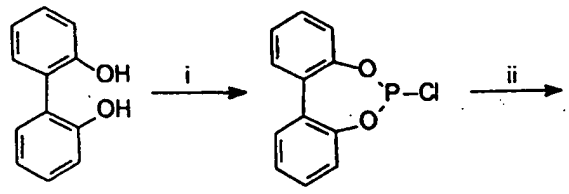
Schem 1

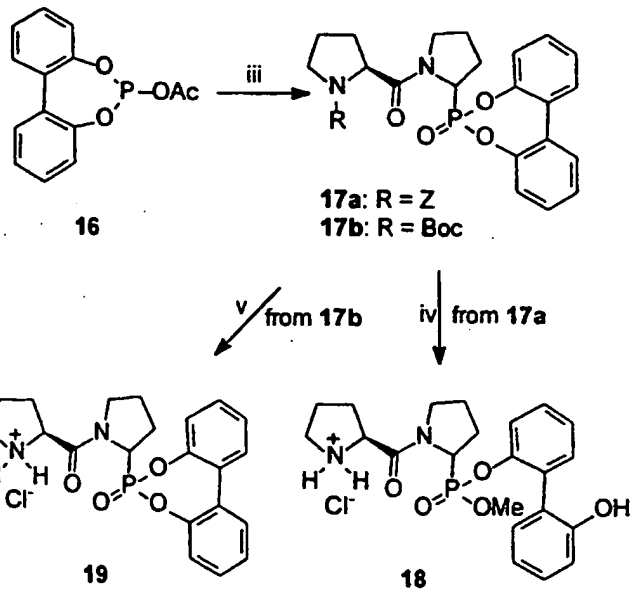


Reagents : i) PCl_3 ; ii) HCl ; iii) HOAc, 90°C, 2 h ; iv) HCl/EtOAc (1 M) ; v) H_2, Pd/C

For 8-11 : R1 = a) H ; b) 4-OMe ; c) 4-OAc (4-OH for 11) ; d) 3-NHAc ; e) 4-NHAc ; f) 4-$NHSO_2Me$; g) 3-$NHCONH_2$; h) 4-(N-Bz-Gly-NH) ; i) 4-(N-Z-Gly-NH) [4-(H-Gly-NH) for 11] ; j) 4-(N-Z-(S)-Ala-NH) [4-(H-(S)-Ala-NH) for 11] ; k) 4-((S)-Pyr-NH) ; l) 4-[(2S)-$MeO_2CCH(NHAc)CH_2$] ; m) 4-COOMe ; n) 4-($CONHCH_2COOEt$) ; o) 4-[$CONH(CH_2)2COOMe$] ; p) 4-($CONH(CH_2)2CH_3$).

Scheme 2





Reagents : i) ; ii) AcOH, Et_3N ; iii) 7, HOAc, 90°C, 2 h ; iv) H2, Pd/C ; v) HCl/EtOAc (1 M).

Figure 1. Correlation between Hammett constant and log k_{calc}.

A good correlation (log k = 4.6504σ + 2.5472, r^2 = 0.82) was observed between the Hammett constant and log kcalc, the most striking divergence being the difference between the unsubstituted (**5**) and the 4-acetylamino (**11e**).

Figure 2. Correlation between Hammett constant and log half-life.

A good correlation (log $t_{1/2}$ = -2.2138σ + 2.3043, r^2 = 0.77) was observed between the Hammett constant and log $t_{1/2}$.

Figure 3. Correlation between log k_{calc} and log half-life.

Figure 4. Plasma DPP IV activity in rabbits treated with **11n**. "R7" and "R8" are rabbits no. 7 and 8.

Figure 5. Residual plasma DPP IV activity in rats treated subcutaneously on days 0 to 5 with **11e**. The indications "R1", "R2", "R3" and "R4" identify the various rats.

A good correlation (log $t_{1/2}$ = -0.4051(log k) + 3.2899, r^2 = 0.75) was observed between log half-life and log k. Comp unds **11i**, **11j**, **18** and **19** were left out. Compounds **11i** and **11j** have a consid rable shorter half-life than could be expected, presumably because they suffer from another metabolism, extra to the phosphonate ester hydrolysis (see discussion). Compounds **18** and **19** cannot be compared directly to the other diaryl phosphonate esters.

EXAMPLES

EXAMPLE 1

Synthesis of a series of protected and unprotected prolylpyrrolidine diaryl phosphonates

The synthesis of a series of prolylpyrrolidine diaryl phosphonates started with the coupling of 4-aminobutyraldehyde diethyl acetal to N-protected proline, activated as mixed anhydride with isobutyl chloroformate, as published previously (Belyaev, A. et al. A New Synthetic Method for Proline Diphenyl Phosphonates. Tetrahedron Lett. 1995, 36, 3755-3758). The acetal **6** was hydrolysed with HCl, and the crude aldehyde **7** was treated with the corresponding triaryl phosphite **9** in acetic acid to give diastereoisomeric mixtures of the protected prolylpyrrolidine diaryl phosphonate **10** (Belyaev et al., (1995), supra) (**Scheme 1**). Deprotection using standard methods afforded the final compounds **11**.

For the preparation of substituted phenyl phosphonates, it was necessary to prepare some commercially unavailable phenols. The 4-methylsulfonyl-aminophenol **8f** was prepared from the corresponding sulfonylchloride and 4-aminophenol. Likewise, the 4-acylaminophenols **8h-k** were prepared by condensation of the corresponding carboxylic acid with 4-aminophenol using the mixed anhydride method. N-acetyl-L-tyrosine methyl ester (**8l**) was prepared as described (Jackson, E. L. O-p-Toluenesulfonyl-L-tyrosine, its N-acetyl and N-benzoyl Derivatives. J. Am. Chem. Soc. 1952, 74,

837-838). The glycine derivativ **8n** was obtained after condensation of glycine thyl ester with 4-hydroxybenzoic acid using diphenylphosph ryl azide (DPPA). The synthesis of the 4-hydroxybenzoic acid amides **8o** and **8p** was accomplished by coupling of 4-acetoxybenzoic acid with the corresponding amine using the mixed anhydride method followed by mild alkaline hydrolysis of the phenyl esters **12** and **13** (Büchi, G.; Weinreb, S. M. Total Syntheses of Aflatoxins M1 and G1 and an Improved Synthesis of Aflatoxin B1. J. Am. Chem. Soc. 1971, 93, 746-752). The triaryl phosphites **9** were then synthesised from the corresponding substituted phenols **8** and phosphorous trichloride.

Cyclic N-protected 2,2'-biphenyl derivatives **17a** and **17b** were prepared by reacting 2,2'-biphenylacetyl phosphite (**16**) with aldehyde **7a** or **7b** in acetic acid (**Scheme 2**). Attempted removal of benzyloxycarbonyl (Z) protection from phosphonate **17a** by hydrogenolysis with a Pd/C catalyst in methanol resulted in the opening of the dioxaphosphepan ring to give the mixed methyl aryl ester **18**. Deprotection of the Boc-derivative **17b** with HCl/EtOAc yielded free 2,2'-biphenyl phosphonate **19**.

Generally, with this method we obtained a mixture of diastereoisomers of the phosphonates **10**. To obtain the pure diastereoisomers of **5**, it was necessary to introduce a trityl protection after removal of the Z-protection, which resulted in easily separable isomers (**10a**(S,R) and **10a**(S,S)). We suppose that the most active isomer has the R configuration at the carbon atom next to phosphorus, and this was confirmed by comparison of the relative mobility on TLC, optical activity, ^{1}H-NMR spectrum and biological activity with that of (S)-Ile-(R)-ProP(OPh)2. The configuration of this reference compound was unambiguously determined with X-ray crystallography (Belyaev et al., (1995), supra).

Experimental:

General. 4-methoxyphenol (**8b**), 1-(3-hydroxyphenyl)urea (**8g**), methyl 4-hydroxybenzoate (**8m**), triphenyl phosphite (**9a**), 2,2'-biphenol (**14**), L-proline, L-tyrosine, 4-aminophenol, glycine, L-pyroglutamic acid, 4-hydroxybenzoic acid, glycine ethyl ester hydrochloride, ß-alanine and all common chemicals were purchased from Acros Chimica N.V., Belgium. 3-Acetamidophenol (**8d**), 4-aminobutyraldehyde diethyl acetal, PCl_3, diphenylphosphoryl azide (DPPA) and 4-acetoxybenzoic acid were obtained from Sigma-Aldrich Chemie BV, Belgium. 4-Acetamidophenol (paracetamol) (**8e**) was obtained from Sterling Organics Ltd., England. Purity of all new synthesised compounds were checked by TLC, ^{1}H-NMR, ^{13}C-NMR or MS. The final products were checked by TLC, ^{1}H-NMR, ^{13}C-NMR, FAB-MS and(or) elemental analysis. Thin-layer chromatography was performed with POLYGRAM(r) SIL G/UV254 plates precoated with silica gel (Machinery-Nagel GmbH, Germany) using EtOAc-petroleum ether, EtOAc-MeOH or n-BuOH-AcOH-H_2O (4:1:1) mixtures as eluent. Silica gel H, 5-40 µm (Fluka, Switzerland) was used for preparative vacuum column chromatography. The NMR spectra were recorded on a Varian EM360L or a Brucker Avance DRX 400 spectrometer. Mass-spectra were recorded on a VG 70-SEQ spectrometer. Optical rotation was measured on a Perkin-Elmer 241 polarimeter. Melting points were determined on a Digital Melting Points Apparatus (Electrothermal) and are uncorrected. Z- and Boc-protected amino acids were prepared according to standard procedures (Bodanszky, M. & Bodanszky, A. In The Practice of Peptide Synthesis, 2nd ed.; Springer-Verlag: Berlin Heidelberg, 1994; pp 11-18 and 28-29) using Z-Cl and $(Boc)_2O$, respectively. ß-Alanine methyl ester was synthesised by treatment of the amino acid in methanolic HCl similarly to described procedure (Bodanszky & Bodanszky (1994), supra). The preparation of 4-(Z-(S)-prolyl)aminobutyraldehyde diethyl acetal (**6a**) was described earlier (Belyaev et al., (1995), supra).

2-Chlor -(dibenzo[d;f]-1,3,2-dioxaphosphepan) (15) was prepared as described (Veriznikov, L.V. & Kirpichnikov, P. A. Synthesis f th o,o'-Biphenylphosphinic Acid Esters. Zh. Obshch. Khim. 1967, 37, 1355-1358).

4-(Boc-(S)-prolyl)aminobutyraldehyde diethyl acetal (6b).

To a solution of Boc-(S)-Proline (10 mmol, 2.14 g) in dry $CHCl_3$ (20 mL), Et_3N (10 mmol, 1.4 mL) was added with stirring at -10°C followed by isobutyl chloroformate (10 mmol, 1.31 mL). After 20 min, 4-aminobutyraldehyde diethyl acetal (10 mmol, 1.73 mL) was added and the mixture was stirred overnight (-10°C to room temperature). Chloroform was removed, the residue was distributed between EtOAc (100 mL) and water (100 mL), the organic layer was dried ($MgSO_4$) and purified by column chromatography. Yield 2.96 g (83%): oil; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.0-2.4 (m, 23H, 3- and 4-CH_2 (Pro), CH_2CH_2, C(CH_3)3, CH_3), 2.9-3.9 (m, 9H, 5-CH_2, NCH_2, OCH_2 and (EtO)2CH), 4.25 (m, ^{1}H, 2-CH), 4.5 (t, ^{1}H, NH).

4-Methylsulfonylaminophenol (8f).

To a suspension of 4-aminophenol (200 mmol, 21.8 g) in MeOH (250 mL), CH_3SO_2Cl (100 mmol, 7.75 mL) was added at 10-15°C with stirring. The resulting solution was stirred for 1 h at room temperature and evaporated. The residue was suspended in 1N HCl (250 mL), the solid was filtered, washed with water and dried in vacuum over NaOH. Yield 13.6 g (81%): mp 165-166°C; ^{1}H-NMR (DMSO) δ (ppm) 2.85 (s, 3H, CH_3S), 6.70 (d, $2H_{arom}$), 7.10 (d, $2H_{arom}$), 8.70 (s, ^{1}H, OH), 8.85 (s, ^{1}H, NH).

Synthesis of 4-acylaminophenols 8h-k

General procedure.

To a solution of carboxylic acid (100 mmol) in DMF (100 mL), Et_3N (100 mmol, 14 mL) was added at -10°C, followed by isobutyl chloroformate (100 mmol, 13 mL). After 0.5 h at this temperature, 4-aminophenol (115 mmol,

12.5 g) was added and the mixture was stirred overnight (-10°C to room temperature). After dilution with 1N HCl (500 mL), th precipitate was collected by filtration, and washed on the filter with water, $NaHCO_3$ solution, water and, finally, ether. The product was dried and, if necessary, recrystallized from an appropriate solvent.

N2-Benzoyl-N-(4-hydroxyphenyl)glycinamide (8h).

Recrystallized from EtOH-acetone. Yield 35%: mp 244-245°C; ^{1}H-NMR (DMSO) δ (ppm) 4.05 (d, 2H, CH_2CO), 6.5-8.0 (m, $9H_{arom}$), 8.7 (m, ^{1}H, NH), 9.15 (s, ^{1}H, OH), 9.75 (s, ^{1}H, NH).

N2-Benzyloxycarbonyl-N-(4-hydroxyphenyl)glycinamide (8i).

Yield 44%: mp 184-185°C; ^{1}H-NMR (DMSO) δ (ppm) 3.80 (d, 2H, CH_2CO), 6.5-7.5 (m, 10H, $9H_{arom}$ and NH), 8.85 (s, ^{1}H, OH), 9.40 (s, ^{1}H, NH).

N2-Benzyloxycarbonyl-N-(4-hydroxyphenyl)-(S)-alaninamide (8j).

Yield 73%: mp 97-100°C; ^{1}H-NMR (DMSO) δ (ppm) 1.36 (d, 3H, CH_3), 4.29 (m, ^{1}H, CH of L-Ala), 5.00 (s, 2H, CH_2), 6.00 (m, ^{1}H, NH), 6.35-7.38 (m, 9H, H_{arom}) 8.69 (br s, ^{1}H, OH), 9.24 (br s, ^{1}H, NHCO)

N-(4-Hydroxyphenyl)-(S)-pyroglutamylamide (8k).

Yield 64%: mp 303-305°C dec; ^{1}H-NMR (DMSO) δ (ppm) 1.7-2.7 (m, 4H, CH_2CH_2), 4.2 (m, ^{1}H, CHCO), 6.65 (d, $2H_{arom}$), 7.40 (d, $2H_{arom}$), 7.8 (s, ^{1}H, NH), 9.1 (br s, ^{1}H, OH), 9.7 (s, ^{1}H, NH).

N-(4-Hydroxybenzoyl)glycine ethyl ester (8n).

To a solution of 4-hydroxybenzoic acid (100 mmol, 13.8 g) in DMF (70 mL), H-Gly-OEt.HCl (100 mmol, 14 g) was added followed by DPPA (100 mmol, 21.6 mL) and Et_3N (200 mmol, 28 mL) with stirring at 0°C. The mixture was stirred overnight (0°C to room temperature), Et_3N.HCl was

removed by filtration and the filtrate was evaporated to half of the initial volume. The residue was mixed with 5% $NaHCO_3$ (200 mL) and extracted with EtOAc (500 mL). The organic layer was washed with brine, dried ($MgSO_4$) and evaporated until crystallisation. The mixture was diluted with ether (100 mL), and the crystals were collected. Yield 9 g (40%): mp 205-209°C; ^{1}H-NMR (DMSO) δ (ppm) 1.25 (t, 3H, CH_3), 4.05 (m, 4H, CO_2CH_2 and $COCH_2N$), 6.8 (d, $2H_{arom}$), 7.75 (d, $2H_{arom}$), 8.35 (tr, ^{1}H, NH), 9.7 (s, ^{1}H, OH).

Synthesis of 4-hydroxybenzoic acid amides 8o and 8p

General procedure.

To a solution of 4-acetoxybenzoic acid (100 mmol, 18 g) in THF (250 mL), Et_3N (100 mmol, 14 mL) was added followed by isobutyl chloroformate (100 mmol, 13 mL) with stirring at -10°C. After 15 min the corresponding amine (free base or HCl salt) was added followed by Et_3N (100 mmol, 14 mL) in case of a hydrochloride. The mixture was stirred overnight (-10°C to room temperature), the solid was removed by filtration, the filtrate was evaporated and the residue crystallised from an ether-hexane mixture.

4-Acetoxybenzoic acid amide 12, 13 thus obtained (70 mmol) was dissolved in MeOH (240 mL),

H_2O (90 mL) was added followed by saturated $NaHCO_3$ solution (120 mL). An excess of $NaHCO_3$ precipitated. The heterogeneous mixture was stirred for 4 h at room temperature, methanol was removed in vacuum, the residue was extracted with EtOAc (250 mL), the organic layer was washed with 1N HCl, brine, then dried ($MgSO_4$) and evaporated. The residue crystallised from ether or was purified on a silica gel column.

N-(4-Acetoxybenzoyl)-ß-alanine methyl ester (12).

Yield 69%: mp 84-88°C; ^{1}H-NMR ($CDCl_3$) δ (ppm) 2.35 (s, 3H, $COCH_3$), 2.6 (t, 2H, CO_2CH_2), 3.7 (m, 5H, NCH_2 and CO_2CH_3), 7.15 (m, 3H, $2H_{arom}$ and NH), 7.75 (d, $2H_{arom}$).

N-n-Propyl-4-acetoxybenzoylamide (13).

Yield 73%: mp 94-98°C; ^{1}H-NMR ($CDCl_3$) δ (ppm) 0.9 (t, 3H, CH_3), 1.6 (m, 2H, CH_2), 2.25 (s, 3H, $COCH_3$), 3.4 (m, 2H, NCH_2), 7.1 (m, 3H, $2H_{arom}$ and NH), 7.8 (d, $2H_{arom}$).

N-(4-Hydroxybenzoyl)-β-alanine methyl ester (8o).

Yield 65%: mp 124-126°C; ^{1}H-NMR (DMSO) δ (ppm) 2.55 (t, 2H, CO_2CH_2), 3.7 (m, 5H, NCH_2 and CO_2CH_3), 6.9 (m, 3H, $2H_{arom}$ and NH), 7.55 (d, $2H_{arom}$), 8.7 (br s, ^{1}H, OH).

N-n-Propyl-4-hydroxybenzoylamide (8p).

Yield 78%: oil; ^{1}H-NMR (DMSO) δ (ppm) 1.1 (t, 3H, CH_3), 1.55 (m, 2H, CH_2), 3.3 (m, 2H, NCH_2), 6.9 (m, 3H, $2H_{arom}$ and NH), 7.65 (d, $2H_{arom}$), 8.5 (br s, ^{1}H, OH); MS (FAB+) m/z 180 $(M+H)^+$.

Synthesis of triaryl phosphites 9

General procedure.

To a stirred solution of the corresponding phenol (90 mmol) and Et_3N (90 mmol, 12.6 mL) in dry DMF (30 mL), a solution of PCl_3 (30 mmol, 2.62 mL) in dry $CHCl_3$ (10 mL) was added dropwise at 0°C. The mixture was stirred overnight (0°C to room temperature), diluted with $CHCl_3$ (500 mL) and the resulting solution was washed with water (2x500 mL). The organic layer was dried ($MgSO_4$), evaporated and the residue, eventually together with the product insoluble in both layers, was purified by column chromatography with an EtOAc - methanol or an EtOAc - petroleum ether mixture as eluent.

Tris(3-acetamidophenyl) phosphite (9d).

Yield: 42%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 2.1 (s, 9H, $COCH_3$), 6.7-7.7 (m, $12H_{arom}$), 9.7 (s, 3H, NH).

Tris(4-acetamidophenyl) phosphite (**9e**).

Yield 37%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 2.1 (s, 9H, $COCH_3$), 6.9-7.7 (dd, $12H_{arom}$), 9.2 (s, 3H, NH).

Tris(4-methylsulfonylaminophenyl) phosphite (**9f**).

Yield 60%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 2.95 (s, 9H, SO_2CH_3), 7.2 (m, $12H_{arom}$), 9.15 (s, 3H, NH).

Tris(3-ureylphenyl) phosphite (**9g**).

Yield 30%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 5.85 (m, 6H, $CONH_2$), 6.5-7.5 (m, $12H_{arom}$), 8.65 (s, 3H, NH).

Tris[4-(N-benzoylglycylamino)phenyl] phosphite (**9h**).

Yield 90%: mp 210°C (dec.); ^{1}H-NMR (DMSO) δ (ppm) 4.1 (d, 6H, $COCH_2N$), 6.9-8.0 (m, $27H_{arom}$), 8.5 (m, 3H, NH), 9.9 (s, 3H, NH).

Tris[4-(N-benzyloxycarbonylglycylamino)phenyl] phosphite (**9i**).

Yield 76%: mp 173°C (dec.); ^{1}H-NMR (DMSO) δ (ppm) 3.85 (d, 6H, $COCH_2N$), 5.05 (s, 6H, CH_2Ph), 6.5-8.0 (m, 30H, NH and H_{arom}), 9.8 (br s, 3H, NH).

Tris[4-(N-benzyloxycarbonyl-(S)-alanylamino)phenyl] phosphite (**9j**).

Yield: 85%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 1.42 (d, 9H, CH_3), 4.29 (m, 3H, CH), 5.01(s, 6H, CH_2Ph), 6.45-7.15 (d, 3H, NH), 7.25 (m, 27H, H_{arom}), 9.76 (br s, 3H, NH).

Tris[4-((S)-pyroglutamylamino)phenyl] phosphite (**9k**).

Yield 31%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 1.8-2.8 (m, 12H, CH_2CH_2), 4.25 (m, 3H, COCHN), 7.1 (d, $6H_{arom}$), 7.65 (d, $6H_{arom}$), 8.25 (s, 3H, NH), 10.3 (s, 3H, NH).

Tris{4-[-(S)-(2-methoxycarbonyl-2-acetamido)ethyl]phenyl} phosphite (91).

Yield 73%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 2.0 (s, 9H, $COCH_3$), 3.1 (d, 6H, $ArCH_2$), 3.7 (s, 9H, CO_2CH_3), 4.8 (m, 3H, COCHN), 6.6 (d, 3H, NH), 7.1 (br s, $12H_{arom}$).

Tris(4-methoxycarbonylphenyl) phosphite (9m).

Yield: 57%: oil; ^{1}H-NMR ($CDCl_3$) δ (ppm) 3.95 (s, 9H, CO_2CH_3), 6.65-8.05 (m, $12H_{arom}$).

Tris{4-[(ethoxycarbonyl)methylaminocarbonyl]phenyl} phosphite (9n). Yield 70%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.3 (t, 9H, CH_3), 3.9-4.3 (m, 12H, $COCH_2N$ and OCH_2), 6.7-8.0 (m, 15H, NH and H_{arom}).

Tris{4-[2-(methoxycarbonyl)ethylaminocarbonyl]phenyl} phosphite (9o).

Yield 71%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 2.65 (t, 6H, CO_2CH_2), 3.7 (m, 15H, NCH_2 and CO_2CH_3), 7.1 (d, $6H_{arom}$), 7.9 (m, 9H, H_{arom} and NH).

Tris[4-(n-propylaminocarbonyl)phenyl] phosphite (9p).

Yield 54%: mp 207°C; ^{1}H-NMR (DMSO) δ (ppm) 1.1 (t, 9H, CH_3), 1.6 (m, 6H, CH_2), 3.25 (m, 6H, CH_2N), 7.15 (d, $6H_{arom}$), 8.1 (m, 9H, NH and H_{arom}).

Synthesis of N-Boc- or N-Z-protected diaryl 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonates 10

General procedure.

Diethyl acetal 6a or 6b (10 mmol) was dissolved in a mixture of THF (60 mL) and 0.5N HCl (30 mL, 15mmol) with stirring at room temperature. After 2 h ether (90 mL) was added with stirring and the mixture was neutralised with an excess of solid $NaHCO_3$ (ca. 5 g). The organic layer was separated, dried ($MgSO_4$) and evaporated. The residue (crude aldehyde 7a or 7b) was dissolved in acetic acid (30 mL) together with the corresponding

triaryl phosphite (9, 10 mmol) and the resulting solution was stirred at 85-90°C for 1.5-2 h. Aft r cooling to ro m temperature, acetic acid was evaporated, the residue was dissolved in chl roform (250 mL), washed with wat r (200 mL), saturated $NaHCO_3$ solution (100 mL) and water (100 mL). The organic layer was dried ($MgSO_4$), evaporated and the residue was purified by column chromatography with an ethyl acetate-petroleum ether or ethyl acetate-methanol mixture as eluent.

Di(3-acetamidophenyl) 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (10d).

Yield 31%: solid foam: ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.0-2.7 (m, 8H, 3-CH_2 and 4-CH_2), 2.1 (s, 6H, CH_3CO), 4.0-3.2 (m, 4H, 5-CH_2), 4.3-5.1 (m, 2H, 2-CH), 5.1 (br s, 2H, CH_2Ph), 6.6-7.7 (m, 13H_{arom}), 9.1 (s, 2H, NH); MS (FAB+) m/z 649 $(M+H)^+$.

Di(4-acetamidophenyl) 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (10e).

Yield 60%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.6-2.7 (m, 8H, 3-CH_2 and 4-CH_2), 2.1 (s, 6H, CH_3CO), 3.3-3.8 (m, 4H, 5-CH_2), 4.3-5.1 (m, 2H, 2-CH), 5.1 (br s, 2H, CH_2Ph), 6.8-7.5 (m, 13H_{arom}), 8.8 (s, 2H, NH); MS (FAB+) m/z 649 $(M+H)^+$.

Di(4-methylsulfonylaminophenyl) 1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (10f).

Yield 66%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.2-2.7 (m, 8H, 3-CH_2 and 4-CH_2), 2.8 (s, 6H, CH_3SO_2), 3.3-3.8 (m, 4H, 5-CH_2), 4.3-5.2 (m, 4H, 2-CH and CH_2Ph), 6.8-7.4 (m, 13H_{arom}), 8.0 (s, 2H, NH); MS (FAB+) m/z 721 $(M+H)^+$.

Di(3-ureylphenyl) 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (10g).

Yield 8%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.3-2.4 (m, 8H, 3-CH_2 and 4-CH_2), 2.8-3.8 (m, 4H, 5-CH_2),

4.3-5.2 (m, 4H, 2-CH and CH_2Ph), 5.6 (m, 4H, NH_2), 6.8-7.5 (m, $13H_{arom}$), 8.65 (br s, 2H, NH); MS (FAB+) m/z 651 $(M+H)^+$.

Di[4-(N-benzoylglycylamino)phenyl]-1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (10h).

Yield 47%: solid foam; ^1H-NMR ($CDCl_3$) δ (ppm) 1.2-2.7 (m, 8H, 3-CH_2 and 4-CH_2), 3.2-3.8 (m, 4H, 5-CH_2), 4.0 (m, 4H, NCH_2CO), 4.4-5.0 (m, 2H, 2-CH), 5.1 (br s, 2H, CH_2Ph), 6.6-8.3 (m, 25H, $23H_{arom}$ and NH), 9.5 (br s, 2H, NH); MS (FAB+) m/z 887 $(M+H)^+$.

Di[4-(N-benzyloxycarbonylglycylamino)phenyl]-1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (10i).

Yield 19%: solid foam; ^1H-NMR ($CDCl_3$) δ (ppm) 1.2-2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.2-4.1 (m, 8H, 5-CH_2 and $COCH_2N$), 4.3-5.2 (m, 8H, 2-CH and CH_2Ph), 6.1 (m, 2H, NH), 6.7-7.6 (m, $23H_{arom}$), 8.95 (br s, 2H, NH); MS (FAB+) m/z 947 $(M+H)^+$.

Di[4-(N-benzyloxycarbonyl-(S)-alanylamino)phenyl]-1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (10j).

Yield 40%. mp: 200-203°C; ^1H-NMR ($CDCl_3$) δ (ppm) 1.35 (d, 6H, CH_3), 1.65-2.35 (m, 8H, 3-CH_2 and 4-CH_2), 2.95-3.95 (m, 4H, 5-CH_2), 4.0-4.65 (m, 4H, 2-CH, CH), 5.00 (s, 6H, CH_2Ph), 6.20 (br, 2H, NH), 7.04 (s, 23H, H_{arom}), 9.25 (br, 2H, NH); MS (FAB+) m/z 975 $(M+H)^+$.

Di[4-((S)-pyroglutamylamino)phenyl]-1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (10k).

Yield 25%: mp 170°C (dec.); ^1H-NMR ($CDCl_3$) δ (ppm) 1.2-2.9 (m, 16H, 3-CH_2 and 4-CH_2), 3.55 (m, 4H, 5-CH_2), 4.1 (m, 2H, 5-CH Pyr), 4.3-5.2 (m, 4H, 2-CH and CH_2Ph), 6.7-8.1 (m, 15H, NH and $13H_{arom}$), 9.55 (br s, 2H, NH); MS (FAB+) m/z 787 $(M+H)^+$.

Di{4-[-(S)-(2-methoxycarbonyl-2-acetamido)ethyl]phenyl} 1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**101**).

Yield 57%: solid f am; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.3-2.7 (m, 8H, 3-CH_2 and 4-CH_2), 1.9 (s, 6H, $COCH_3$), 3.1 (d, 4H, CH_2Ar), 3.7 (s, 6H, $COOCH_3$), 3.2-3.9 (m, 4H, 5-CH_2), 4.5-5.0 (m, 4H, 2-CH), 5.1 (br s, 2H, CH_2Ph), 6.1 (d, 2H, NH), 7.0 (s, 8H_{arom}), 7.3 (br s, 5H, C_6H5); MS (FAB+) m/z 821 $(M+H)^+$.

Di{4-[(ethoxycarbonyl)methylaminocarbonyl]phenyl} 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (**10n**).

Yield 32%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.2-2.7 (m, 8H, 3-CH_2 and 4-CH_2), 1.3 (t, 6H, CH_3), 3.2-3.9 (m, 4H, 5-CH_2), 4.0 (m, 4H, NCH_2CO), 4.2 (q, 4H, OCH_2), 4.3-5.1 (m, 2H, 2-CH), 5.1 (br s, 2H, CH_2Ph), 6.9-7.8 (m, 15H, 13H_{arom} and NH); MS (FAB+) m/z 793 $(M+H)^+$.

Di{4-[2-(methoxycarbonyl)ethylaminocarbonyl]phenyl} 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (**10o**).

Yield 22%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.2-2.8 (m, 8H, 3-CH_2 and 4-CH_2), 2.65 (t, 4H, CH_2CO_2), 3.2-3.8 (m, 8H, 5-CH_2 and CH_2N), 3.75 (s, 6H, CO_2CH_3), 4.3-5.2 (m, 4H, 2-CH and CH_2Ph), 6.7-7.9 (m, 15H, 13H_{arom} and NH); MS (FAB+) m/z 793 $(M+H)^+$.

Di[4-(n-propylaminocarbonyl)phenyl] 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (**10p**).

Yield 4.3%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 0.85 (t, 6H, CH_3), 1.3-2.8 (m, 12H, 3-CH_2, 4-CH_2 and CH_2), 3.1-3.9 (m, 8H, 5-CH_2 and CH_2N), 4.5 (m, ^{1}H, 2-CH Pro), 4.7-5.2 (m, 3H, CH_2Ph and 2-CH ProP), 6.9-8.1 (m, 15H, 13H_{arom} and NH); MS (FAB+) m/z 705 $(M+H)^+$.

Diaryl 1-((S)-prolyl)pyrrolidine-2-phosphonate hydrochlorides (11).

Procedure A.

The diaryl 1-(t-butyloxycarbonyl-(S)-prolyl)-pyrrolidine-2-phosphonate (10) or diaryl 1-(trityl-(S)-prolyl)pyrrolidine-2-phosphonate (10, 5 mmol) was dissolved in a 1M HCl solution in EtOAc (25 mL) and the solution was stirred for 2 h at room temperature. Dry ether (30 mL) was added and the mixture was left in the fridge overnight. If the product crystallised, the crystals were collected by filtration, otherwise the oil was triturated in dry ether. The material obtained was dried in vacuum over NaOH pellets.

Procedure B.

The diaryl 1-((S)-benzyloxycarbonylprolyl)-pyrrolidine-2-phosphonate (10, 2 mmol) was hydrogenated over Pd/C in methanol (50 mL) for 5-6 h. The catalyst was removed by filtration through celite, the filtrate was acidified with 1M HCl/EtOAc (2.2 mL), evaporated and the residue was precipitated with dry ether from methanol (5 mL). The resulting product was dried in vacuum over NaOH pellets.

Diphenyl 1-((S)-prolyl)pyrrolidine-2(R)-phosphonate hydrochloride (11a(S,R)).

A. Yield 81%: mp 175-177°C; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.6-2.8 (m, 8H, 3-CH_2 and 4-CH_2), 3.3-3.9 (m, 4H, 5-CH_2), 4.8 (m, ^{1}H, 2-CH), 5.0 (m, ^{1}H, 2-CH), 7.2 (m, 5H_{arom}), 7.3 (m, 5H_{arom}), 8.1 (br s, ^{1}H, N+H), 10.2 (br s, ^{1}H, N+H); ^{13}C-NMR ($CDCl_3$) δ (ppm) 24.1(4-CH_2), 24.6 (4-CH_2), 26.1 (3-CH_2), 28.8 (3-CH_2), 46.2 (5-CH_2), 47.1 (5-CH_2), 54.5 (d, 2-CH-P, 1J (C-P) = 162 Hz), 59.1 (2-CH), 120.3, 125.2, 129.7, 150.3 (C_{arom}), 168.2 (CO); $[\alpha]D^{20}$ = -100.4° (C_1, $CHCl_3$); Anal. ($C_{21}H_{25}N_2O_4P$ 1.25 HCl) C, H, N.

Diphenyl 1-((S)-prolyl)pyrrolidine-2(S)- phosphonate hydrochloride (**11a**(S,S)).

A. Yield 88%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.7-2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.2-3.9 (m, 4H, 5-CH_2), 4.75 (m, ^{1}H, 2-CH), 4.95 (m, 0.7H, 2-CH), 5.4 (m, 0.3H, 2-CH), 6.9-7.4 (m, 10H_{arom}), 8.6 (br s, ^{1}H, N+H), 10.3 (br s, 0.3H, N+H), 10.6 (br s, 0.7H, N+H); ^{13}C-NMR ($CDCl_3$) δ (ppm) 22.1, 24.0 (4-CH_2), 23.5, 23.9 (4-CH_2), 26.4, 27.7 (3-CH_2), 28.5, 29.1 (3-CH_2), 45.6, 46.2 (5-CH_2), 46.6, 47.1 (5-CH_2), 54.4, 55.3 (d, 2-CH-P, 1J (C-P) = 160 Hz), 58.3, 58.7 (2-CH), 120.1, 125.0, 129.4, 149.5 (C_{arom}), 167.3, 168.4 (CO); $[\alpha]D^{20}$ = 25.9° (C_1, $CHCl_3$); MS (FAB+) m/z 401 $(M+H)^+$.

Di(3-acetamidophenyl) 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (**11d**).

B. Yield 83%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 1.6-2.6 (m, 8H, 3-CH_2 and 4-CH_2), 2.1 (s, 6H, $COCH_3$), 3.3-3.8 (m, 4H, 5-CH_2), 4.7 (m, ^{1}H, 2-CH), 4.9 (m, ^{1}H, 2-CH), 6.7-7.6 (m, 8H_{arom}), 8.5 (m, ^{1}H, N+H), 9.7 (m, ^{1}H, N+H), 9.9 (m, 2H, CONHPh); Anal. ($C_{25}H_{31}N_4O_6P$ 1.5 HCl 0.5 H_2O) C, H, N.

Di(4-acetamidophenyl) 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (**11e**).

B. Yield 90%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 1.6-2.6 (m, 8H, 3-CH_2 and 4-CH_2), 2.1 (s, 6H, $COCH_3$), 3.3-3.8 (m, 4H, 5-CH_2), 4.6 (m, ^{1}H, 2-CH), 4.9 (m, ^{1}H, 2-CH), 7.1 (m, 4H_{arom}), 7.6 (m, 4H_{arom}), 8.7 (m, ^{1}H, N+H), 10.0 (m, 2H, CONHPh), 10.2 (m, ^{1}H, N+H); 31P-NMR (DMSO) δ (ppm) 17.48, 17.62; Anal. ($C_{25}H_{31}N_4O_6P$ HCl 2 H_2O) C, H, N.

Di(4-methylsulfonylaminophenyl) 1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11f**).

B. Yield 80%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 1.55-2.5 (m, 8H, 3-CH_2 and 4-CH_2), 2.95 (s, 6H, SO_2CH_3), 3.3-3.8 (m, 4H, 5-CH_2), 4.55 (m, ^{1}H, 2-CH), 4.85 (m, ^{1}H, 2-CH), 7.15 (m, 8H_{arom}), 8.6 (br s, ^{1}H, N+H), 9.79 (s, 2H,

SO_2NHPh), 10.3 (br s, ^1H, N+H); Anal. ($C_{23}H_{31}N_4O_8PS_2$ HCl) C, H, N.

Di(3-ureylphenyl) 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (11g).

B. Yield 90%: solid foam; ^1H-NMR (CD3OD) δ (ppm) 1.4-2.5 (m, 8H, 3-CH_2 and 4-CH_2), 3.3-3.8 (m, 4H, 5-CH_2), 4.5 (m, ^1H, 2-CH), 4.8 (m, ^1H, 2-CH), 6.4-7.2 (m, $6H_{arom}$), 7.4 (m, $2H_{arom}$); MS (FAB+) m/z 517 $(M+H)^+$; Anal ($C_{23}H_{29}N_6O_6P$ HCl 2.3 H_2O) C, H, N.

Di[4-(N-benzoylglycylamino)phenyl]-1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (11h).

B. Yield 87%; solid foam; ^1H-NMR (DMSO) δ (ppm) 1.6-2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.2-3.6 (m, 4H, 5-CH_2), 4.2 (d, 4H, NCH_2CO), 4.5-4.9 (m, 2H, 2-CH), 6.9-7.9 (m, 20H, $18H_{arom}$ and NH), 8.5 (m, ^1H, N+H), 10.0 (s, 2H, NH), 10.2 (m, ^1H,N+H); MS (FAB+) m/z 753 $(M+H)^+$; Anal. ($C_{39}H_{41}N_6O_8P$ HCl 2 H_2O) C, H, N.

Di[4-(N-glycylamino)phenyl]-1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate trihydrochloride (11i).

B. Yield 81%: solid foam; ^1H-NMR (DMSO) δ (ppm) 1.55-2.5 (m, 8H, 3-CH_2 and 4-CH_2), 3.3-3.8 (m, 4H, 5-CH_2), 3.8 (s, 4H, $COCH_2N$), 4.55 (m, ^1H, 2-CH), 4.85 (m, ^1H, 2-CH), 7.15 (m, $4H_{arom}$), 7.65 (m, $4H_{arom}$), 8.35 (br s, 6H, N+H), 8.5 (br s, ^1H, N+H), 10.35 (br s, ^1H, N+H), 11.0 (s, 2H, NH); Anal. ($C_{25}H_{33}N_6O_6P$ 3 HCl 2.5 H_2O) C, H, N.

Di(4-(S)-alanylaminophenyl)-1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate trihydrochloride (11j).

B. Yield 70%; solid foam; ^1H-NMR (D2O) δ (ppm) 1.49 (d, 6H, CH_3), 1.65-2.60 (m, 8H, 3-CH_2 and 4-CH_2), 3.23-3.75 (m, 4H, 5-CH_2), 4.12 (m, 2H, CH of Ala), 7.05 (m, 4H, H_{arom}), 7.35 (m, 4H, H_{arom}); Anal. ($C_{27}H_{37}N_6O_6P$ 3 HCl 0.5 H_2O) C, H; N: calcd, 12.16; found, 11.73.

Di(4-(S)-pyroglutamylaminophenyl)-1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (**11k**).

B. Yield 95%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 1.5-2.4 (m, 16H, 3-CH_2 and 4-CH_2), 3.1-4.2 (m, 6H, 5-CH_2 and 5-CH), 4.5 (m, ^{1}H, 2-CH Pro), 4.85 (m, ^{1}H, 2-CH ProP), 7.1 (m, 4H_{arom}), 7.65 (m, 4H_{arom}), 7.9 (s, 2H, NH), 8.6 (m, ^{1}H, N+H), 10.1 (m, ^{1}H, N+H), 10.3 (s, 2H, NH); Anal. ($C_{31}H_{37}N_6O_8P$ 1.5 HCl 3 H_2O) C, H, N.

Di{4-[-(S)-(2-methoxycarbonyl-2-acetamido)ethyl]phenyl} 1-((S)-prolyl)pyrrolidine-2-phosphonate hydrochloride (**11l**).

B. Yield 85%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 1.6-2.6 (m, 8H, 3-CH_2 and 4-CH_2), 1.9 (s, 6H, $COCH_3$), 3.0 (d, 4H, CH_2Ar), 3.2-3.8 (m, 4H, 5-CH_2), 3.7 (s, 6H, $COOCH_3$), 4.4-4.9 (m, 4H, 2-CH), 7.1 (m, 8H_{arom}), 8.2 (d, 2H, AcNH), 8.7 (m, ^{1}H, N+H), 10.4 (m, ^{1}H, N+H); MS (FAB+) m/z 687 $(M+H)^+$; Anal. ($C_{33}H_{43}N_4O_{10}P$ 1.5 HCl 2 H_2O) C, N; H: calcd, 6.29; found, 5.81.

Di{4-[(ethoxycarbonyl)methylaminocarbonyl]phenyl} 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**11n**).

B. Yield 86%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 1.3 (t, 6H, CH_3), 1.4-2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.2-3.8 (m, 4H, 5-CH_2), 4.0 (m, 4H, NCH_2CO), 4.2 (q, 4H, OCH_2), 4.6 (m, ^{1}H, 2-CH), 4.9 (m, ^{1}H, 2-CH), 7.3 (m, 4H_{arom}), 7.9 (m, 4H_{arom}), 8.7 (m, 4H, N+H and NH); MS (FAB+) m/z 659 $(M+H)^+$; Anal. ($C_{31}H_{39}N_4O_{10}P$ HCl 1.5 H_2O) C, H, N.

Di{4-[2-(methoxycarbonyl)ethylaminocarbonyl]phenyl} 1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11o**).

B. Yield 79%: solid foam; ^{1}H-NMR (DMSO) δ (ppm) 1.2-2.8 (m, 8H, 3-CH_2 and 4-CH_2), 2.65 (t, 4H, CH_2COO), 3.2-3.9 (m, 8H, 5-CH_2 and CH_2N), 3.8 (s, 6H, $COOCH_3$), 4.7 (m, ^{1}H, 2-CH), 4.9 (m, ^{1}H, 2-CH), 7.55 (m, 4H_{arom}), 8.15 (m, 4H_{arom}), 8.55 (m, 4H, N+H and NH).

Di[4-(n-propylaminocarbonyl)phenyl] 1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (11p).

B. Yield 80%: solid f am; ^{1}H-NMR (CD3OD) δ (ppm) 0.95 (t, 6H, CH_3), 1.6 (m, 4H, CH_2), 1.7-2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.2-3.9 (m, 8H, 5-CH_2 and CH_2N), 4.65 (m, ^{1}H, 2-CH Pro), 5.1 (m, ^{1}H, 2-CH ProP), 7.3 (m, 4H_{arom}), 7.85 (m, 4H_{arom}); ^{13}C-NMR (CD3OD) δ (ppm) 11.7 (CH_3), 23.5 (CH_2), 25.0 (4-CH_2), 25.8 (4-CH_2), 27.3 (3-CH_2), 29.7 (3-CH_2), 42.8 (NCH_2), 47.4 (5-CH_2), 48.3 (5-CH_2), 55.9 (d, 2-CH-P, 1J (C-P) = 161 Hz), 60.6 (2-CH), 121.4, 130.4, 133.1, 153.7 (C_{arom}), 168.8 (CO), 169.2 (CO).

2,2'-Biphenylacetyl phosphite (16).

A solution of chloroanhydride 15 (0.152 mol, 38 g) in dry THF (50 mL) was added dropwise to a solution of AcOH (0.152 mol, 9.1 mL) and Et_3N (0.152 mol, 21.3 mL) in dry THF (100 mL) with stirring at 0°C. The cooling was removed and the mixture was stirred at room temperature for 3 h. The solid was filtered off and the filtrate was evaporated. The crude product was used in the next step without further purification. Yield 40 g (96%): viscous oil; ^{1}H-NMR ($CDCl_3$) δ (ppm) 2.1 (s, 3H, CH_3CO), 6.9-7.5 (m, 8H, H_{arom}).

2,2'-Biphenyl 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (17a).

Prepared according to the general procedure for diaryl phosphonates with acetal 6a and phosphite 16. Yield 27%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.2-2.9 (m, 8H, 3-CH_2 and 4-CH_2), 3.1-4.0 (m, 4H, 5-CH_2), 4.55 (m, ^{1}H, 2-CH Pro), 4.85 (m, ^{1}H, 2-CH ProP), 5.2 (br s, 2H, CH_2Ph), 7.1-7.85 (m, 15H_{arom}); MS (EI) m/z (relative intensity) 532 (M, 40), 397 (20), 300 (26), 232 (29), 215 (41), 204 (45), 168 (42), 160 (74), 91 (100), 70 (48).

2,2'-Biphenyl 1-(t-butyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (17b).

Prepared according to the general procedure for diaryl phosphonates with acetal **6b** and phosphite **16**.

Yield 24%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.3-2.55 (m, 8H, 3-CH_2 and 4-CH_2), 1.5 (s, 9H, CH_3), 3.35-3.9 (m, 4H, 5-CH_2), 4.55 (m, ^{1}H, 2-CH), 4.9 (m, ^{1}H, 2-CH), 6.95-7.75 (m, 8H, H_{arom}); MS (FAB+) m/z 499 $(M+H)^+$.

2-(2'-Hydroxyphenyl)phenyl methyl 1- (S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (18).

Prepared from the Z-protected derivative **17a** using procedure "B" for diaryl phosphonates.

Yield 92%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.5-2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.1-3.9 (m, 7H, 5-CH_2 and OCH_3), 4.4-4.8 (m, 2H, 2-CH Pro and 2-CH ProP), 6.85 (br s, ^{1}H, PhOH), 7.1-7.6 (m, 8H_{arom}), 8.4 (m, ^{1}H, N+H), 9.6 (m, ^{1}H, N+H); ^{13}C-NMR ($CDCl_3$) δ (ppm) 25.7 (4-CH_2), 26.1 (4-CH_2), 28.2 (3-CH_2), 30.5 (3-CH_2), 48.1 (5-CH_2), 48.6 (5-CH_2), 55.6 (OCH_3), 55.6 (2-CH-P, 1J (C-P) = 142 Hz), 60.8 (2-CH), 117.8, 121.0, 126.3, 130.1, 132.5, 133.8 149.7, 156.2 (C_{arom}), 168.9 (CO); MS (FAB+) m/z 431 $(M+H)^+$; Anal. ($C_{22}H_{27}N_2O_5P$ HCl H_2O) C, H; N: calcd, 5.78; found, 6.26.

2,2'-Biphenyl 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (19).

Prepared from the Boc-protected derivative **17b** using procedure "A" for diaryl phosphonates.

Yield 81%: solid foam; ^{1}H-NMR ($CDCl_3$) δ (ppm) 1.3-2.55 (m, 8H, 3-CH_2 and 4-CH_2), 3.35-3.75 (m, 4H, 5-CH_2), 4.75 (m, 2H, 2-CH), 7.0-7.5 (m, 8H, H_{arom}), 10.9 (m, 2H, N+H); ^{13}C-NMR ($CDCl_3$) δ (ppm) 24.4 (4-CH_2), 24.9 (4-CH_2), 26.7 (3-CH_2), 28.7 (3-CH_2), 46.6 (5-CH_2), 51.9 (5-CH_2), 53.4 (2-CH-P, 1J (C-P) = 151 Hz), 59.2 (2-CH), 116.9, 120.9, 121.7, 121.9, 125.8, 126.6, 128.4, 129.0, 130.3, 131.5, 147.9 (C_{arom}), 167.8 (CO); MS (FAB+) m/z 399

$(M+H)^+$; Anal. ($C_{21}H_{23}N_2O_4P$ 1.4 HCl 1.1 H_2O) C, H; N: calcd, 5.97; found, 5.43.

EXAMPLE 2

Synthesis of a series of diaryl phosphonates with an alanine as residue aa

Diphenyl alanine phosphonates were synthesized following the principle described by Oleksyszyn et al., Synthesis 1979, 985-986. The method was used with acetaldehyde for the preparation of the alanine analogue, diphenyl 1-benzyloxycarbonylaminoethane phosphonate. After deprotection, the mixed anhydride method was used to couple diphenyl 1-aminoethane phosphonate to various amino acids : glycine, L-alanine, L-valine, L-isoleucine, L-phenylalanine and L-proline.

Experimental :

Preparation of the diphenyl alanine phosphonates

Diphenyl 1-(N-benzyloxycarbonyl) aminoethane phosphonate (X)

A mixture of triphenyl phosphate (100 mmol, 31.03 g), acetaldehyde (150 mmol, 6.61 g - freshly distilled), benzylcarbamate (100 mmol, 15.10 g) and glacial acetic acid (15 ml) was stirred for about 30 minutes until the exothermic reaction subsides.

The mixture was heated at 80-85°C for one hour and the volatile products were removed on a rotary evaporator under reduced pressure with heating on a boiling water bath. The oily residue was dissolved in methanol (180 ml) and left overnight for crystallization at -10°C. The crystalline ester was collected by filtration, redissolved in a small amount of hot chloroform (30-40 ml) and recrystallization occurred by adding a 4-fold volume of methanol.

Yield: 46%; m.p.: 117-118°C IR (KBr): 3260 (NH); 3050, 3020 (CHar); 2960, 2920 (CHalif); 1720 (C=O); 1240 (P=O); 1200 (P-O); 740, 690 (C_6H_5); ^{1}H-NMR ($CDCl_3$, 60

MHz): 1.55 (3H, dd, J_{PH}= 17.5 Hz, J_{HH}= 7 Hz, CH_3); 4.53 (1H, m, CH); 5.10 (2H, s, OCH,); 5.37 (1H, br, NH); 6.93-7.23 (10H, m, Har); 7.23-7.37 (5H, m, Har)

Diphenyl 1-aminoethane phosphonate hydrobromide (Y)

Compound X (10 mmol, 4.11 g) was dissolved in a 45% solution of hydrogen bromide in acetic acid (5 ml). After-one hour at room temperature the solvent and volatile products were removed under reduced pressure on a boiling water bath. The oily residue was triturated with dry ether. The crystals were filtered and dried over sodium hydroxide in a vacuum oven to give the pure hydrobromides.

Yield: 95%; m.p.: 148.5°C; IR (KBr): 3100-2700 (NH_3^+); 1230 (P=O); 1200 (P-O); 760, 680 (C_6H_5); ^{1}H-NMR ($CDCl_3$ + d6-DMSO, 60 MHz): 1.77 (3H, dd, J_{PH} = 17.0 Hz, J_{HH} = 7 Hz, CH_3); 4.05 (1H, m, CH); 6.97-7.30 (10H, m, Har)

Diphenyl 1(R,S)-[N-benzyloxycarbonyl-L-phenylalanyl]-aminoethane phosphonate, Diphenyl 1(R,S)-[N-benzyl-oxycarbonyl-L-prolyl]aminoethane phosphonate (T, Q)

General procedure for the mixed anhydride coupling with isobutyl chloroformate.

The Z-protected amino acid (5 mmol) was dissolved in dry tetrahydrofuran (25 ml) and cooled to -150°C in an ice-sodium chloride bath. N-Methyl-morpholine (5 mmol, 0.51 g) and isobutyl chloroformate (5 mmol, 0.68 g) were added to this solution and stirring was continued for 10 minutes. A solution of Y (5 mmol, 1.79 g) and triethylamine (5 mmol, 0.51 g) in dimethyl-formamide (10 ml), was introduced dropwise to the stirring mixture, keeping the temperature below -100°C.

After one hour in a cold water bath, the reaction mixture was allowed to warm to room temperature. The hydrochlorides of N-methylmorpholine and triethyl-amine were removed by filtration and washed with tetrahydrofuran. The combined filtrate and washings were concentrated on a rotary evaporator. The residue was

dissolv d in ethyl acetate (75 ml) and water (25 ml) and the organic phase was washed with 5% sodium bicarb nat (25 ml), 0.5N hydr g n chlorid solution (25 ml) and saturated sodium chloride solution (25 ml), dried over sodium sulphate and evaporated to dryness under reduced pressure.

T: Yield: 84%; oil; IR(KBr):3280(NH); 3060(CHar); 2960, 2930, 2880 (CHalif); 1720-1660 (C=O); 1250(P=O); 1180(P-O);760,685(C_6H_5)

^{1}H-NMR($CDCl_3$,60MHz):1.33 (3H,dd,J_{PH}=17.5Hz, J_{HH}=7.0Hz,CH_3); 2.80-3.10(2H,m,βH); 4.27-4.73(2H,m,αH,CH); 5.05(2H,m,CH_2); 5.53(2H,br,NH); 6.87-7.30(20H,m,Har)

Q: Yield: 79%; oil; IR(KBr):3280(NH); 3060(CHar); 2960,2930,2880(CHalif); 1720-1660 (C=O);1260(P=O);1180(P-O);760,685(C_6H_5)

^{1}H-NMR($CDCl_3$,60MHz):1.43(3H,dd,J_{PH}=18.0Hz, J_{HH}=7.0Hz,CH_3);1.75-2.10(4H,m,βH,yH);3.35-3.70(2H,m,δH); 4.25-4.50(1H,m,αH);4.85(1H,m,CH); 5.13(2H,s,CH_2);7.10-7.40(15H,m,Har)

Diphenyl 1(R,S)-L-phenylalanyl aminoethane phosphonate, diphenyl 1(R,S)-L-prolyl aminoethane phosphonate (K, L)

General procedure for removal of the Z-group by hydrogenolysis

The Z-protected dipeptide phosphonates T and Q (1.5 mmol) were dissolved in dry methanol (20 ml) and acetic acid (0.7 ml). After the addition of palladium on charcoal (10%, 0.25 g), the air above the solution was displaced with nitrogen and hydrogen was led over the solution for 3 hours. Before removing the solvent under reduced pressure, the flask was flushed with nitrogen for 10 minutes. The residue was dissolved in dry chloroform (15 ml) and 0.5N hydrogen chloride in ethyl acetate was added slowly to the stirring solution. Removal of the solvent under reduced pressure and co-evaporation with 0.5N hydrogen chloride in ethyl acetate (10 ml) afforded a white foam, which was triturated with dry ether. After

2 days at -10°C, the crystals were filtered off and the hygroscopic product was kept in a desiccator.

K: Yield: 80%; oil; IR:3240(NH);3100-2800(NH_3^+); 1690(C=O);1250(P=O);1180(P-O);750,680(C_6H_5)
^{1}H-NMR($CDCl_3$,250MHz):1.15-1.50(3H,dd,J_{PH}=17Hz, J_{HH}=6.7Hz,CH_3); 3.16,3.43(2H,m,βH); 44.46-4.58; 4.46-4.70(3H,m,αH,CH,NH); 6.98-7.34 (15H,m,Har);8.30-8.52(2H,-br,NH_2)

L: Yield 75%; oil; IR:3240(NH):3100-2800(NH_3^+); 1690(C=O);1250(P=O); 1180(P-O);760,680(C_6H_5)
^{1}H-NMR($CDCl_3$,d6-DMSO,250MHz):1.37-1.65 (3H,m,CH_3); 1.77-2.03 (4H,m,βH,yH); 3.38-3.51(2H,m,δH); 4.31(1H,m,NH); 4.55(1H,m,αH); 4.77-4.88(1H,m,CH);7.13-7.31(10H,m,Har)

EXAMPLE 3

DPP IV inhibition and stability of the inhibitors

In a previous study (Lambeir, A. M. et al. Dipeptide-derived Diphenyl Phosphonate Esters: Mechanism-based Inhibitors of Dipeptidyl Peptidase IV. Biochim. Biophys. Acta 1996, 1290, 76-82) on the role of the P-2 amino acid in dipeptide diphenyl phosphonates, the present inventors showed that proline in this position gives one of the most potent inhibitors. A major advantage of **5** (the mixture of diastereoisomers of **11a**) was its greater stability in human citrated plasma compared to the other dipeptide derivatives. The stability of **5** in plasma equals the stability in buffer, whereas for the other compounds the stability in plasma was reduced compared to buffer. This reflects the relative stability of a Pro-Pro amino acid sequence to proteolytic breakdown, and indicates that the decrease in activity of **5** is mainly caused by hydrolysis of the phosphonate ester.

To increase the inhibit ry activity while retaining the stability several substituents w re introduced on the phenyl rings that act as leaving groups.

The influence of electron-donating or -withdrawing substituents on enzyme inhibition and stability was investigated as shown in Table 1. All compounds were irreversible inhibitors of DPP IV, probably due to the formation of a phosphonylated serine at the active site of the enzyme. The inactivation rate constants were calculated from experimental IC_{50} values and were in reasonable agreement with the measured inactivation rate constants, where available.

A good correlation was observed between the electron-withdrawing properties of the substituent and its activity (Figure 1). Introduction of an electron-donating substituent (4-OH, **11c**) decreases potency, whereas an electron-withdrawing substituent (4-methoxycarbonyl, **11m**) increases potency.

Quite unexpectedly, a striking divergence was observed in the correlation between the Hammett constant and the inhibitory activity for compounds **11e** (4-acetylaminophenyl) and **11f** (4-methylsulfonyl-aminophenyl). Both the compounds (**11e**) and (**11f**) strongly inhibited DPP IV. These compounds have similar Hammett constants compared to the diastereoisomeric mixture of **5**, but are nevertheless about 100 times more potent.

Introduction of other acylamino substituents was investigated. The 4-glycylamino (**11i**) and the 4-alanylamino (**11j**) have a comparable activity, but are considerably less stable in plasma than the 4-acetylamino (**11e**) substituted diphenyl phosphonate.

The half-life of the inhibitors seems to also correlate with the Hammett constant (Figure 2). Compounds with similar electronic properties (e.g. **5** and **11e**) have a similar stability in plasma.

The O-methyl derivative (**18**) is almost as p tent and stable as **5**.

The correlation betw en electronic properti s and inhibitory activity and between electronic properties and stability results in an inverse correlation between inhibitory activity and stability (Figure 3). This means that a more active compound is also more unstable. Therefore, the higher potency (IC_{50} = 0.4 μM) and equal stability ($t_{1/2}$ = 320 min) of the paracetamol substituted phenyl phosphonate (**11e**) is a major improvement to the unsubstituted **5**.

Experimental:

DPP IV was purified from human seminal plasma as described previously (De Meester et al., J. Immunol. Meth. 189, 99-105 (1996)). Enzymatic activity was measured at 37°C in a Spectramax 340 (Molecular Devices) microtiterplate reader using Gly-Pro-p-nitroanilide (Sigma) as a chromogenic substrate. The reaction was monitored at 405 nm and the initial rate was determined between 0 and 0.25 absorbance units. The reaction mixture contained 2 mM substrate, approximately 1 mU of DPP IV, 40 mM TRIS-HCl buffer, pH 8.3, and a suitable amount of inhibitor (ranging between 0 an 10 mM) in a total volume of 0.2 ml. Activity measurements were routinely performed in duplicate.

The IC_{50} value is defined as the concentration of inhibitor required to reduce the DPP IV activity to 50% after a 15 min pre-incubation with the enzyme at 37°C before addition of the substrate. Inhibitor stock solutions (100 mM) were prepared in DMSO or phosphate buffer, pH 7.4, depending on the solubility of the compound, and stored at -20°C. Stock solutions were diluted with 50 mM TRIS-HCl buffer, pH 8.3, as required, immediately before the experiment. Since the compounds described in this paper completely inactivate DPP IV following pseudo-first order kinetics, the IC_{50} value is inversely correlated with the second order rate constant

of inactivation (Lambeir et al., (1996), supra). For a simpl pseudo-first ord r inactivation process, the activity after incubation with inhibitor (vi) varies with the inhibitor concentration (i) as described in the following equation: vi = vo * e-k.i.t, where vo is the activity in absence of inhibitor, k is the second order rate constant of inactivation and t is the time. Since at $i = IC_{50}$ by definition vi = 1/2 vo, it follows from the equation that $k = \ln 2/(ti*IC_{50})$ where ti = 15 min. These calculated k values are listed in Table 1.

Table 1. Potency and stability of DPP IV inhibitors

	R_1	Configuration at Pro^P C(2)	IC_{50} (μM)	k_{obs} ($M^{-1}s^{-1}$)	$t_{1/2}$ (min) in plasma
	H	R,S	32 (n=1)	$2.4*10^1$	250 ± 10
11a	H	R	15 ± 3 (n=3)	$5.1*10^1$	300 ± 30
11a	H	S	$>10^4$		
11d	3-AcNH	R,S	0.8 ± 0.1 (n=2)	$9.6*10^2$	220 ± 30
11e	4-AcNH	R,S	0.4 ± 0.2 (n=5)	$1.9*10^3$	320 ± 140
11f	4-$MeSO_2NH$	R,S	0.40 ± 0.02 (n=2)	$1.9*10^3$	150 ± 30
11g	3-H_2NCONH	R,S	2.3 ± 0.3 (n=2)	$3.3*10^2$	210 ± 80
11h	4-(*N*-Bz-Gly-NH)	R,S	0.7 ± 0.3 (n=2)	$1.1*10^3$	93 ± 3
11i	4-(H-Gly-NH)	R,S	0.5 ± 0.1 (n=2)	$1.5*10^3$	28 ± 3
11j	4-(H-(S)-Ala-NH)	R,S	0.6 ± 0.2 (n=2)	$1.3*10^3$	8 ± 1
11k	4-((S)-Pyr-NH)	R,S	5.0 ± 0.8 (n=2)	$1.5*10^2$	170 ± 30
11l	4-[(2S)-$MeO_2CCH(NHAc)CH_2$]	R,S	1.4 ± 0.5 (n=4)	$5.5*10^2$	190 ± 150
11m	4-MeO_2C	R,S	0.016 ± 0.004 (n=2)	$4.8*10^4$	19 ± 1
11n	4-(EtO_2CCH_2NHCO)	R,S	0.023 ± 0.007 (n=2)	$3.3*10^4$	35 ± 2
11o	4-[$MeO_2C(CH_2)_2NHCO$]	R,S	0.036 ± 0.006 (n=4)	$2.1*10^4$	26 ± 1
11p	4-[$CH_3(CH_2)_2NHCO$]	R,S	0.03 ± 0.01 (n=2)	$2.6*10^4$	12 ± 1
18	P(OMe)(OC_6H_4(2-OH-C_6H_4))	R,S	47 ± 18 (n=2)	$1.6*10^1$	140 ± 80
19	P-2,2'-biphenyl	R,S	31 ± 6 (n=2)	$2.5*10^1$	6 ± 1

IC_{50} values were determined after 15 min pre-incubation with DPP IV at 37 °C. The listed values are the average of n independent measurements ± the standard deviation. Functional stability in plasma was estimated by fitting the inverse of the apparent IC_{50} values versus time with a single exponential decay. The half-life is listed ± the standard error of fit.

For some inhibitors (5, 11a, 11b, 11d, 11e, 11h, 11n) the inactivation rate constant was determined from the time course of inhibition as described before (Lambeir et al., (1996), supra).

The functional stability of the inhibitors was estimated by measuring the inhibitory potency (apparent IC_{50}) of a 1 mM dilution of the compounds in citrated human plasma at 3 or 4 time points between 0 and 300 min at 37°C. Fitting the inverse of the apparent IC_{50} values versus time with a single exponential decay gives the half-lives reported in table 1.

EXAMPLE 4

Inhibition of prolyl oligopeptidase (PO) by protected prolylpyrrolidine diaryl phosphonates

The inhibitory capacity of various compounds of the invention was evaluated. The results are shown in table 2.

Table 2

Potency of the compounds 10 as PO inhibitors. IC_{50} values are expressed in μM

compound	R1 or R2	IC_{50}
10d	3-AcNH	10
10e	4-AcNH	21
10f	4-$MeSO_2NH$	10
10h	4-(N-Bz-Gly-NH)	12
10i	4-(N-Z-Gly-NH)	15
10j	4-(N-Z-Ala-NH)	>500
10l	4-[(2S)-MeO_2CCH-(NHAc)CH_2]	20
10k	4-((S)-Pyr-NH	9

10n	4-(EtO_2CCH_2NHCO)	5
10	4-[MeO_2C-$(CH_2)_2NHCO$]	2.8
10p	4-[$CH_3(CH_2)_2NHCO$]	3.2

Experimental:

Prolyl oligopeptidase was purified from human platelets and the enzyme activity was measured using Z-Gly-Pro-AMC (4.4 mM) as the substrate in a K-phosphate buffer 100 mM, pH 7.5 containing 1 mM EDTA, 1 mM dithiothreitol and 1 mM NaN_3. The incubation was carried out during 20 min at 37°C. The reaction was stopped by the addition of 5 volumes 1.5 M acetic acid. Fluorescence was measured at 370 and 440 nm as excitation and emission wavelengths respectively. The inhibitors were added at concentrations varying between 1 μM and 1 mM.

EXAMPLE 5

Inhibition of Dipeptidyl peptidase II by dipeptide-derived diaryl phosponate esters with an ala in position aa

The compounds Pro-AlaP(OPh)2 and Phe-AlaP(OPh)2 inhibited DPP II in vitro and the IC_{50} values calculated were 1.5 mM for Pro-AlaP(OPh)2 and 0.8 mM for Phe-AlaP(OPh)2.

Experimental:

Dipeptidyl peptidase II was semi-purified from rabbit kidney and its activity was determined by the hydrolysis of Lys-Ala-4-MeO-2-NA 1.4 mM (Sigma, L-2270) in 100 mM acetate buffer, pH 5.5 containing 2 mM EDTA. After incubation during 20 minutes at 37°C, the reaction was stopped by the addition of 10 fold excess sodium-acetate pH 3.6. The fluorescence of the formed 4-MeO-2-NA was measured at 340 and 425 nm as excitation and emission wavelengths respectively. For the determination of the IC_{50} values the compounds were tested

at a series of concentrations ranging from 1 μM to 5 mM during incubation.

EXAMPLE 6

In vitro cytotoxicity and efficacy of prolylpyrrolidine diaryl phosphonates in human peripheral blood mononuclear cells (PBMC)

Based on inhibition potency, stability in plasma and synthesis efficiency, compounds **11e** and **11n** were selected for further in vitro and in vivo studies. Both compounds were evaluated in human peripheral blood mononuclear cells (PBMC) and did not show cytotoxicity in freshly isolated mononuclear cells or phytohemagglutinin stimulated blasts when concentrations up to 100 μM were used. Under these circumstances, more than 90 % of the DPP IV activity in cell lysates as well as in supernatants was inhibited. This is in contrast with the results obtained for the active diastereoisomer of the unsubstituted diphenyl phosphonate (**11a**), where no satisfactory inhibition of DPP IV activity could be reached without cytotoxic effects on PBMC cultures. The compounds **11e** and **11n** are therefore promising tools for further studies on cellular level. The irreversible mechanism of inhibition overcomes the rather limited stability of the compounds in biological media.

Experimental:

Peripheral blood mononuclear cells were isolated from buffy coats (obtained from the blood transfusion center of Antwerp). After dilution (1/4) in phosphate buffer saline (PBS), cells were layered onto Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden) and centrifuged at room temperature at 550 x g during 20 min. The interfaces were collected and washed 3 times in RPMI-1640. Finally the cells were resuspended at $1x10^6$ cells/mL in RPMI-1640 containing 10 % heat-inactivated foetal calf serum, and antibiotics (penicillin/streptomycin) (Gibco). These freshly isolated

cells were used immediately for inhibitor studi s or first stimulat d with phytohaemagglutinin (Murex diagnostics) at 1μg/mL during 3 days at 37°C in a 5 % CO_2 humidified incubator. Inhibitor (stock solution at -80°C in phosphate buffer, diluted ex tempore in RPMI-1640) or vehicle alone was added to the cells ($5x10^6$/test) at different concentrations. After overnight incubation at 37°C, an aliquot was taken for cytotoxicity evaluation by 0.4 % trypan blue exclusion. The remaining cells were washed 3 times in PBS and the final cell pellet was solubilised in 200 μL PBS containing 1 % v/v Triton X-100 and 100 KIU/mL aprotinin (Bayer) and centrifuged during 10 min at 20000 x g. Supernatants were used immediately for enzyme assay and protein determination by the Bradford micro-assay. Specific activities (U/g protein) are compared and the % inhibition is given toward control samples without inhibitor.

EXAMPLE 7

DPP IV inhibition in vivo

It was reported previously (De Meester et al., Biochem. Pharmacol. 54, 173-179 (1997)) that a single intravenous injection of 5 (0.3-5 mg/kg) in rabbits caused a decrease in plasma DPP IV activity with more than 80 % and it took more than 20 days for complete recovery.

In rats, a comparable dose per weight intravenously did not result in a sufficient inhibition of circulating DPP IV, because adequate inhibition could not be reached without severe systemic toxicity. However, a combination of subcutaneous and intraperitoneal injections of **11a** allowed us to bring plasma DPP IV to less than 15 % of pre-treatment values. The observation that monotherapy with the diastereoisomeric mixture of **11a** (5) not only significantly prolonged graft acceptance upon alloantigen challenge, but occasionally also caused systemic toxicity and more often l cal ulcerations,

stimulated the in vivo testing of **11e** and **11n** in rabbits, rats and mice.

In rabbits, the higher in vivo potency f **11n** compared to **11e** was also observed. The IC_{50} values for inhibition of plasma DPP IV in rabbits upon single intravenous injection was below 20 µg/kg for **11n** and was around 0.2 mg/kg for **11e**. In this species single intravenous injection of 0.2 mg/kg of **11n** inhibited plasma DPP IV activity for more than 90 % during at least 24 h, without side effects (see figure 4).

In rats, **11e** as well as **11n** could keep plasma DPP IV activity below 10 % of pre-treatment values by daily subcutaneous injection of 50 mg/kg (initial dose 100 mg/kg), without any sign of acute systemic or local toxicity. Figure 5 depicts residual plasma DPP IV activity in rats treated subcutaneously on days 0 to 5 with **11e**.

Different administration routes in rats were examined for compound **11n**: oral as well as subcutaneous as intraperitoneal and intrarectal routes of administration allowed an in vivo inhibition of DPP IV. The intraperitoneal route being the least efficient i.e. no 50% inhibition after 24h while for the other routes DPP IV activity was still very low (<35% of initial value) 1 day after a single dose (all routes 50 mg/kg).

In mice, a pharmacologically useful inhibition was only obtained with **11n** and not with **11a** and **11e**. The molecular basis for the large interspecies differences in efficiency remains to be elucidated.

Experimental:

Male New Zealand white rabbits (2.5-3.5 kg), Wistar rats (250-350 g) and Swiss mice (23-36 g) were allowed to adjust to their environment for at least 7 days. They received standard diet and water ad lib. Test compounds were dissolved in 50 mM phosphate buffer pH 7.4 at concentrations ranging from 10-100 mg/mL and stored in aliquots at -80°C and were thawed ex tempore. Rabbits

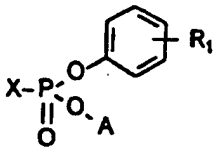
r ceived a single slow intravenous bolus injection of test compound or vehicle alone in the marginal ear v in. Blood was sampl d from the central ear artery. Rats and mice were injected subcutaneously or intraperitoneally. Rat blood samples were obtained under anesthesia (Forene) from the vena femoralis by puncture after incision of the skin. The mice were bled by orbita puncture after induction of anesthesia with pentobarbital. After clotting, blood samples were centrifuged (3000 x g, 10 min) and the resulting sera were stored at -80°C until assayed for DPP IV activity.

The above Examples show that all compounds tested were irreversible inhibitors. A good correlation was observed between the electron-withdrawing properties of the substituents and the inhibition of DPP IV. The methoxycarbonyl and alkylaminocarbonyl substituted derivatives were the most potent inhibitors with IC_{50} values around 20 nM and inactivation rate constants around 3000 M^{-1} s^{-1}. The same correlation was also observed between the electron-donating properties of the substituents and the stability in plasma. The most potent inhibitors are also the most unstable compounds. A notable exception is the good stability of the 4-acetylaminophenyl phosphonate ester (**11e**, $t_{1/2}$ = 320 min), together with a higher potency than could be expected (IC_{50} = 0.4 μM, k = 1900 M^{-1} s^{-1}). Therefore, this compound together with the very potent **11n** were further investigated <u>in vitro</u> and <u>in vivo</u>. These inhibitors showed no cytotoxicity in human peripheral blood mononuclear cells in concentrations up to 100 μM. The IC_{50} values of **11e** and **11n** for inhibition of plasma DPP IV in rabbits upon single intravenous injection were around 0.2 mg/kg and below 20 μg/kg respectively. The compounds also showed no acute systemic or local toxicity, as was observed with the unsubstituted compound **5**.

Due to the higher stability of **11** compared to **11n**, it is believed that di(4-acetamidophenyl) 1-(S)-prolylpyrrolidine-2-(R,S)-phosphonate (**11e**) is a major improvement. The advantage of this compound compared to the pyrrolidine-2-nitrile reversible inhibitors is its long-lasting irreversible inhibition. Moreover, it is more stable than the frequently used boronic acid inhibitors.

CLAIMS

1. Compounds having a modulating activity n serine proteas s and having the general formula



(I)

wherein

- A is ---R2 or H or C_1-C_6 alkyl or halogenoalkyl, except perfluoroalkyl,
- the phenyl group is mono-, di- or trisubstituted with R1 or R2;
- X is a peptide- or amino acid-derived moiety;
- A and the phenyl group substituted with R1 may optionally form a biphenyl diester;
- all R1 substituents and R2 substituents are each independently selected from the group consisting of:

a) C_1-C_6 acylamino;

b) aroylamino, optionally substituted at the o- and/or p- and/or m- position with alkyl, in particular C_1-C_6 alkyl, and/or a halogen;

c) C_1-C_6 alkylsulfonylamino;

d) arylsulfonylamino, optionally substituted at the o- and/or p- and/or m- position with alkyl, in particular C_1-C_6 alkyl, and/or a halogen;

e) α-aminoacylamino wherein the α-aminoacyl represents a side chain blocked or unblocked α-amino acid residue with the L, D or DL configuration at the α-carbon atom selected from the group consisting of:

alanine, methionine, methionine sulfoxide, arginine, homoarginine, phenylalanine, aspartic acid, proline, hydroxyproline, asparagine, serine, cysteine, threonine, histidine, glycine, tyrosine, glutamic acid, pyroglutamic acid, tryptophan, glutamine, valine, norvaline,

isoleucine, lysin , leucine, norleucine, thioproline, homoproline, 1,2,3,4-tetrahydro-is quinoline-3-carboxylic acid (Tic), 2,3-dihydroindol-2-carboxylic acid, α-naphtylglycine, α-phenylglycine, 4-amidinophenylglycine, 4-phenylproline, 4-amidinophenylalanine, O-benzyl tyrosine, omega-acetyl lysine, α-aminobutyric acid, citrulline, homocitrulline, ornithine, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, homoserine, 4-dehydroproline, penicillamine β-(2-thienyl)alanine, NH_2-CH(CH_2CHEt_2)-COOH, α-aminoheptanoic acid, NH_2-CH(CH_2-1-naphthyl)-COOH, NH_2-CH(CH_2-2-naphthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH[CH-(cyclohexyl)$_2$]-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH[CH-(cyclopentyl)$_2$]-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH[CH-(cyclobutyl)$_2$]-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, NH_2-CH[CH-(cyclopropyl)$_2$]-COOH, 5,5,5-trifluoroleucine, hexafluoroleucine, (S)-azetidine-2-carboxylic acid, (S)-pipecolic acid, (S)-oxazolidine-4-carboxylic acid, (R)-thiazolidine-4-carboxylacid (L-thioproline), sarcosine;

f) residue selected from the group consisting of 3-aminobenzoic acid; ε-aminocaproic acid, β-alanine;

g) Y-NH-CO-NH-;

h) Y'O_2CCH(NHCO-Y)-CH_2-;

i) Y'NHCO-;

j) CH_3-O-CO-Y'-NH-CO-;

k) CH_3-CH_2-O-CO-Y'-NH-CO-;

wherein Y is C_1-C_6 alkyl, aryl or H and Y' is C_1-C_6 alkyl,

and pharmaceutically acceptable salts thereof.

2. Compounds according to claim 1, wherein X is a moiety of the general formula $(AA)_p$-aa-,

wherein:

p indicates that there may be 0, 1, 2, 3, 4 r 5 residues AA, which can be the same or different within one molecule;

AA and aa are α-amino carboxylic acids with in α position an optionally substituted C_1-C_6 alkyl or aryl or aralkylmoiety;

and pharmaceutically acceptable salts thereof.

3. Compounds according to claim 1, wherein X is a moiety of the general formula $(AA)_p$-aa-,

wherein:

p indicates that there may be 0, 1, 2, 3, 4 or 5 residues AA, which can be the same or different within one molecule;

AA and aa are selected from the group consisting of:

alanine, methionine, methionine sulfoxide, arginine, homoarginine, phenylalanine, aspartic acid, proline, hydroxyproline, asparagine, serine, cysteine, threonine, histidine, glycine, tyrosine, glutamic acid, pyroglutamic acid, trytophan, glutamine, valine, norvaline, isoleucine, lysine, leucine, norleucine, thioproline, homoproline, 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid (Tic), 2,3-dihydroindol-2-carboxylic acid, α-naphtylglycine, α-phenylglycine, 4-amidinophenylglycine, 4-phenylproline, 4-amidinophenylalanine, O-benzyl tyrosine, omega-acetyl lysine, α-aminobutyric acid, citrulline, homocitrulline, ornithine, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, homoserine, 4-dehydroproline, penicillamine β-(2-thienyl)alanine, NH_2-CH(CH_2CHEt_2)-COOH, α-aminoheptanoic acid, NH_2-CH(CH_2-1-naphthyl)-COOH, NH_2-CH(CH_2-2-naphthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH[CH-(cyclohexyl)$_2$]-COOH,

NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH[CH-(cyclop ntyl)$_2$]-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH[CH-(cyclobutyl)$_2$]-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, NH_2-CH[CH-(cyclopropyl)$_2$]-COOH, 5,5,5-trifluoroleucine, hexafluoroleucine, (S)-azetidine-2-carboxylic acid, (S)-pipecolic acid, (S)-oxazolidine-4-carboxylic acid, (R)-thiazolidine-4-carboxylacid (L-thioproline), 3-aminobenzoic acid, sarcosine, ϵ-aminocaproic acid, ß-alanine, wherein the alpha amino residue may be side chain blocked or unblocked and has the L, D, or DL configuration at the alpha carbon atom;

and pharmaceutically acceptable salts thereof.

4. Compounds as claimed in claims 2-3, wherein AA is lysine or ornithine, and the amino side chain thereof is involved in an intramolecular covalent bond.

5. Compounds as claimed in claim 1, wherein X is M-$(AA)_p$-aa-

wherein:

p, AA and aa are as defined in claims 2-4; and

M is selected from:

a) the group consisting of optionally substituted -$CONH_2$, -$CSNH_2$, -SO_2NH_2, phenyl-SO_2-, phenyl-CH_2SO_2-, 2-furyl-acryloyl; and

b) the group of protecting groups consisting of:

acetyl, adamantyloxycarbonyl, benzyl-oxycarbonyl, benzoyl, benzyl, t-butoxycarbonyl, t-butyl, 2,4-dinitrophenyl, formyl, fluorenylmethoxycarbonyl, 4-methoxybenzyl, tosyl, trifluoro-acetyl, trityl, phthaloyl, phenyl-alkylcarb nyl, 2-indanylacetyl, 2-

(1,2,3,4-tetrahydronaphtyl)acetyl, 4-(4-benzylphenoxy)alkyl;

and pharmaceutically acceptable salts thereof.

6. Compounds as claimed in claim 1-5, wherein X represents (M-)AA-aa-, wherein (M-) means that M may or may not be present, aa is proline and AA is as defined in claims 2-4.

7. Compounds as claimed in claims 1-5, wherein X represents (M-)AA-aa-, wherein (M-) means that M may or may not be present, and wherein AA and aa are both proline.

8. Compounds as claimed in claims 2-5, wherein aa is alanine, R1 and R2 may, in addition to the definitions in claim 1, further be selected from:

l) H, halogen, NO_2, CN, OH, COOH

m) amino, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino,

n) C_1-C_6 acyl

o) C_1-C_6 alkoxy-CO-

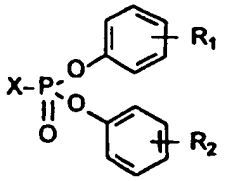
p) C_1-C_6 alkyl-S-,

and pharmaceutically acceptable salts thereof.

9. Compounds as claimed in claim 2-8 wherein at least the AA coupled to aa is proline or phenylalanine and pharmaceutically acceptable salts thereof.

10. Compound as claimed in claim 9, which compound is Phe-Ala-diphenylphosphonate or Pro-Ala-diphenylphosphonate and pharmaceutically acceptable salts thereof.

11. Compounds according to claim 1, wherein the compounds (also indicated as group 1 compounds) have the general formula:



(II)

wherein the R1, R2 and X are as defined in claims 1-9, and pharmaceutically acceptable salts thereof.

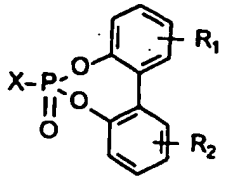
12. Compound as claimed in claim 11 selected from the group consisting of :

- Di(3-acetamidophenyl) 1-(benzyloxycarb nyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonat **(10d)**;
- Di(4-acetamidophenyl) 1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate **(10e)**;
- Di(4-methylsulfonylaminophenyl) 1-(benzyloxy-carbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate **(10f)**;
- Di(3-ureylphenyl) 1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate **(10g)**;
- Di[4-(N-benzoylglycylamino)phenyl]-1-(benzyl-oxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phospho-nate **(10h)**;
- Di[4-(N-glycylamino)phenyl]-1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)- phosphonate **(10i)**;
- Di[4-(N-benzyloxycarbonyl-(S)-alanylamino)phenyl]-1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate **(10j)**;
- Di[4-((S)-pyroglutamylamino)phenyl]-1-(benzyloxy-carbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate **(10k)**;
- Di{4-[-(S)-(2-methoxycarbonyl-2-acetamido)ethyl]-phenyl}1-(benzyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate **(10l)**;
- Di(4-methoxycarbonylphenyl)1-(tert-butyloxycarbonyl-(S)-prolyl)pyrrolidine-2(R,S)-phosphonate **(10m)**;
- Di{4-[(ethoxycarbonyl)methylaminocarbonyl]phenyl} 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate **(10n)**;
- Di{4-[2-(methoxycarbonyl)ethylaminocarbonyl]phenyl} 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate **(10o)**;
- Di[4-(n-propylaminocarbonyl)phenyl] 1-(benzyloxy-carbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate **(10p)**;
- Di(3-acetamidophenyl) 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride **(11d)**;

- Di(4-acetamid phenyl) 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (**11e**);
- Di(4-methylsulfonylaminophenyl) 1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11f**);
- Di(3-ureylphenyl) 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (**11g**);
- Di[4-(N-benzoylglycylamino)phenyl]-1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11h**);
- Di[4-(N-glycylamino)phenyl]-1-((S)-prolyl)-pyrrolidine-2 (R,S)-phosphonate trihydrochloride (**11i**);
- Di(4-(S)-alanylaminophenyl)-1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate trihydrochloride (**11j**);
- Di(4-(S)-pyroglutamylaminophenyl)-1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11k**);
- Di{4-[-(S)-(2-methoxycarbonyl-2-acetamido)ethyl]-phenyl) 1-((S)-prolyl)pyrrolidine-2-phosphonate hydrochloride (**11l**);
- Di{4-[(ethoxycarbonyl)methylaminocarbonyl]phenyl} 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate (**11n**);
- Di{4-[2-(methoxycarbonyl)ethylaminocarbonyl]phenyl} 1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11o**);
- Di[4-(n-propylaminocarbonyl)phenyl] 1-((S)-prolyl)-pyrrolidine-2(R,S)-phosphonate hydrochloride (**11p**);

and pharmaceutically acceptable salts thereof.

13. Compounds according to claim 1, wherein the compounds are 2,2' biphenyl diesters of α-aminoalkyl phosphonic acid (group 2 compounds) having the general formula:



(III)

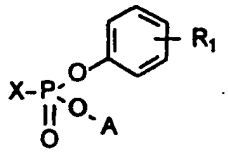
wherein the R1, R2 and X are as defined in claims 1-11, and pharmaceutically acceptable salts thereof.

14. Compounds as claimed in claim 13, selected from the group consisting of:

- 2,2'-Biphenyl 1-(benzyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (17a);
- 2,2'-Biphenyl 1-(t-butyloxycarbonyl-(S)-prolyl)-pyrrolidine-2(R,S)-phosphonate (17b);
- 2,2'-Biphenyl 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride (19).

15. Compound as claimed in claim 14 which compound is 2,2'-Biphenyl 1-((S)-prolyl)pyrrolidine-2(R,S)-phosphonate hydrochloride or a pharmaceutically acceptable salt thereof having an inhibitory activity for DPP IV.

16. Compounds according to claim 1 (group 3 compounds), having the general formula:



(IV)

wherein the R1 is as defined in claim 1, X is as defined in claims 2-5 and A is H or C_1-C_6 alkyl or halogenoalkyl, except perfluoroalkyl, or pharmaceutically acceptable salts thereof.

17. Compound as claimed in claim 16, which compound is

- 2-(2'-Hydroxyphenyl)phenyl methyl 1-(S)-prolyl-pyrrolidine-2(R,S)-phosphonate hydrochloride (18).

18. Compounds as claimed in claims 1-17, wherein the compounds have a modulating activity for DPP IV, for DPP II, for PO, for lysosomal Pro-X carboxy-peptidase or for elastase or for related peptidases with at least 45% amino acid sequence homology with one of these peptidases.

19. Compounds as claimed in claims 1-18 for use as a therapeutical agent.

20. Compounds as claimed in claims 1-18, which compounds may be optionally labeled, for use in the

treatment or prophylaxis of one of the following conditions: inflammati n, organ sp cific or systemic aut -immune diseases, non-malignant disord r of leukocytes and/or immunoglobulins, rejection of cells or tissues after transplantation, tissue destructive and bone degenerative diseases, neuroendocrine dysfunction, glucose-intolerance, obesitas, functional gastrointestinal disorder, abnormal growth or growth retardation, thrombosis and hemorrhage, vascular and cardiopulmonary diseases, neurodegenerative and affective disorders, pain, diseases associated with neoplasia.

21. Compounds as claimed in claims 1-18, which compounds are optionally labeled, for use in diagnostic and research methods such as fluorescence, purification and radio-assays, imaging, in situ histochemical and cytochemical staining.

22. Compounds as claimed in claims 1-18, wherein X is as defined in claims 1-5, wherein R1 and/or R2 is selected from the group consisting of 3-AcNH, 4-AcNH, 4-$MeSO_2NH$, 4-(N-Bz-Gly-NH), 4-(H-(S)-Ala-NH, 4-[(2S)-$MeO_2CCH(NHAc)CH_2$], 4-(S)-Pyr-NH, 4-(EtO_2CCH_2NHCO), 4-[$MeO_2C(CH_2)_2NHCO$], 4-[$CH_3(CH_2)_2NHCO$].

23. Compounds as claimed in claims 1-18, wherein X is as defined in claim 5, wherein R1 and/or R2 is selected from the group consisting of 3-AcNH, 4-AcNH, 4-$MeSO_2NH$, 4-(N-Bz-Gly-NH), 4-(H-(S)-Ala-NH, 4-[(2S)-$MeO_2CCH(NHAc)CH_2$], 4-(S)-Pyr-NH, 4-(EtO_2CCH_2NHCO), 4-[$MeO_2C(CH_2)_2NHCO$], 4-[$CH_3(CH_2)_2NHCO$] and which compounds have an modulating, in particular an inhibitory activity on PO.

24. Use of the compounds as claimed in claims 1-18 for the preparation of a therapeutical composition for inhibiting the activity of serine proteases.

25. Use of the compounds as claimed in claims 1-18 for the preparation of a therapeutical composition for the treatment or prophylaxis of one of the following conditions: inflammation, organ specific or systemic auto-immune diseases, non-malignant disorder of

leukocytes and/or immunoglobulins, rejection of cells or tissues after transplantation, tissue destructive and bone degenerative diseases, neuroendocrin dysfunction, glucose-intolerance, besitas, functional gastrointestinal disorder, abnormal growth or growth retardation, thrombosis and hemorrhage, vascular and cardiopulmonary diseases, neurodegenerative and affective disorders, pain, diseases associated with neoplasia.

26. A pharmaceutical preparation comprising one or more compounds as claimed in claims 1-18 and a suitable excipient, carrier or diluent.

27. A pharmaceutical preparation as claimed in claim 26, further comprising one or more additional therapeutic ingredients.

28. Pharmaceutical preparation as claimed in claim 27 for use in the treatment or prophylaxis of one of the following conditions: inflammation, organ specific or systemic auto-immune diseases, non-malignant disorder of leukocytes and/or immunoglobulins, rejection of cells or tissues after transplantation, tissue destructive and bone degenerative diseases, neuroendocrine dysfunction, glucose-intolerance, obesitas, functional gastrointestinal disorder, abnormal growth or growth retardation, thrombosis and hemorrhage, vascular and cardiopulmonary diseases, neurodegenerative and affective disorders, pain, diseases associated with neoplasia.

29. A method for <u>in vitro</u> modulating protease activity by means of a suitable concentration of a compound as claimed in claims 1-18.

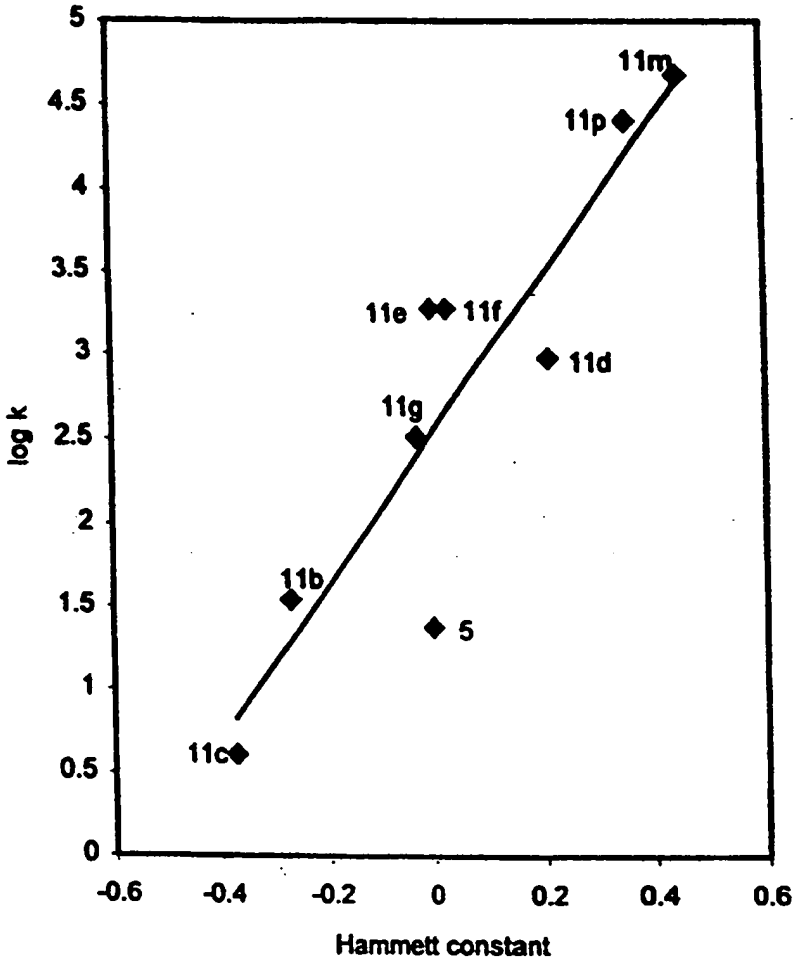
30. Method as claimed in claim 29, wherein the protease activity results in the unwanted degradation of a peptide substrate prior to measurement of the peptide substrate in a peptide assay.

31. A method for <u>ex vivo</u> inhibiting protease activity by means of a suitable concentration of a compound as claimed in claims 1-18.

32. Method as claimed in claim 31, wherein the suitable concentration of the compound is applied to cells or organs for transplantation.

33. A method for in vivo inhibiting protease activity by means of administering to a living organism a suitable amount of a compound as claimed in claims 1-18.

34. A method for treatment or prophylaxis of one of the following conditions: inflammation, organ specific or systemic auto-immune diseases, non-malignant disorder of leukocytes and/or immunoglobulins, rejection of cells or tissues after transplantation, tissue destructive and bone degenerative diseases, neuroendocrine dysfunction, glucose-intolerance, obesitas, functional gastrointestinal disorder, abnormal growth or growth retardation, thrombosis and hemorrhage, vascular and cardiopulmonary diseases, neurodegenerative and affective disorders, pain, diseases associated with neoplasia, comprising administering to a living organism an effective amount of a compound according to claims 1-18.


log k
Hammett constant
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4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
11m
11p
11e
11f
11d
11g
11b
5
11c

Fig. 1

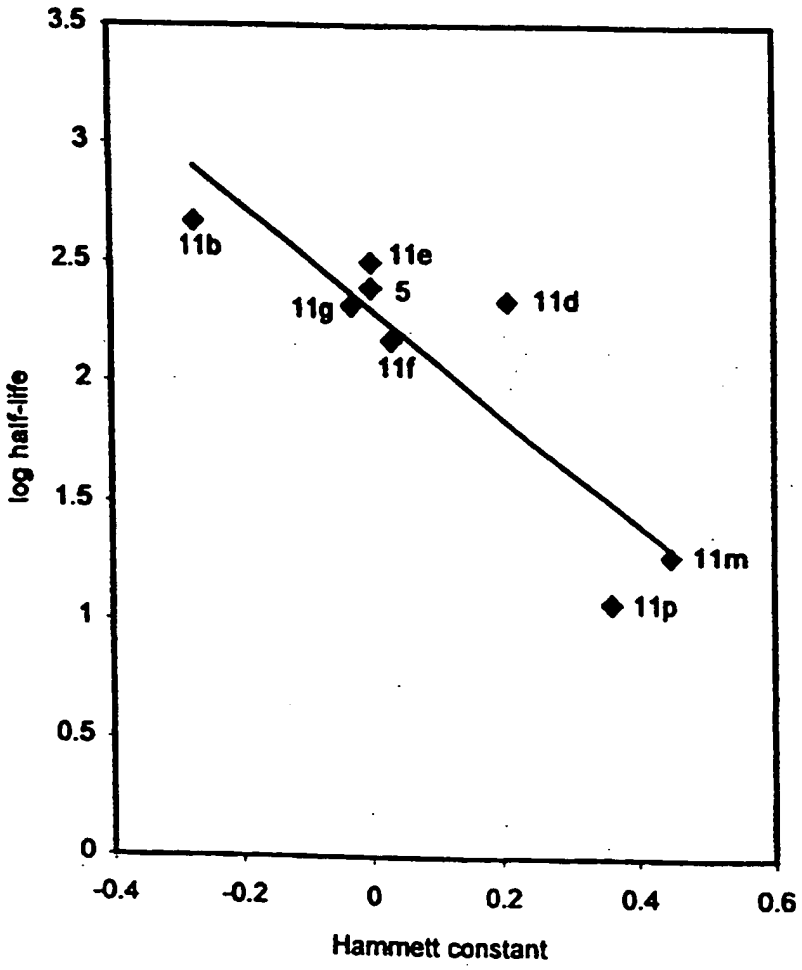
3.5
3
2.5
2
1.5
1
0.5
0
log half-life
-0.4
-0.2
0
0.2
0.4
0.6
Hammett constant
11b
11e
5
11g
11d
11f
11m
11p

Fig. 2

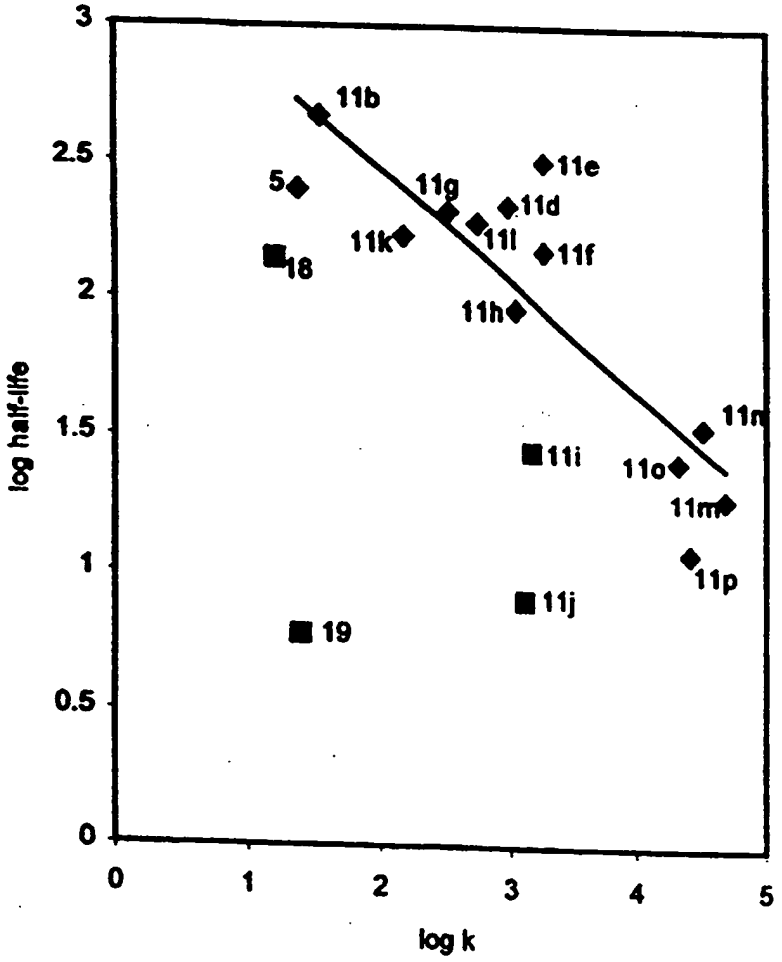
log half-life
log k
0
0.5
1
1.5
2
2.5
3
0
1
2
3
4
5
11b
5
11g
11e
11d
11k
11l
11f
18
11h
11n
11i
11o
11m
11p
11j
19

Fig. 3

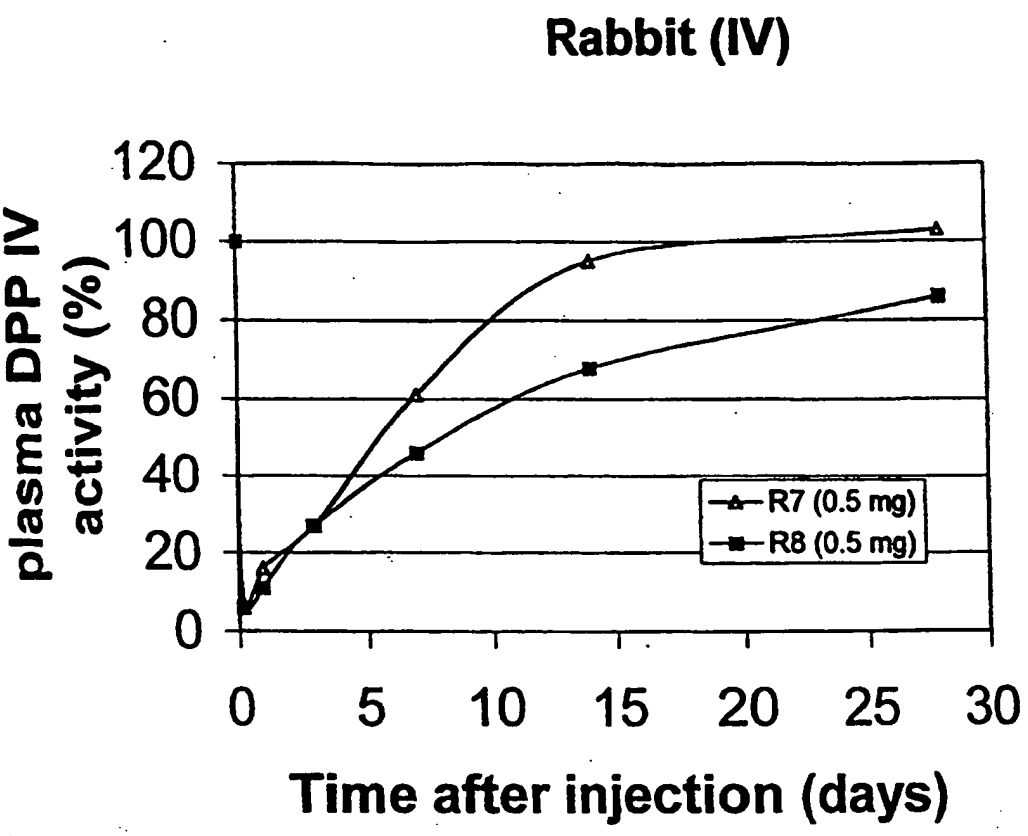
Rabbit (IV)
plasma DPP IV activity (%)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Time after injection (days)
R7 (0.5 mg)
R8 (0.5 mg)

FIG.4

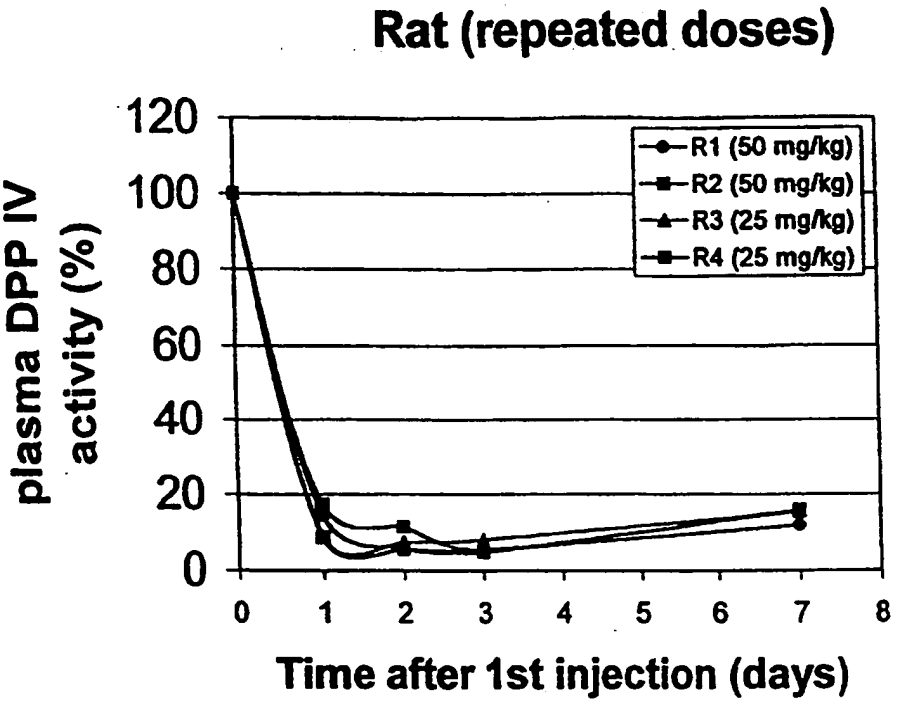
Rat (repeated doses)
plasma DPP IV activity (%)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time after 1st injection (days)
R1 (50 mg/kg)
R2 (50 mg/kg)
R3 (25 mg/kg)
R4 (25 mg/kg)

FIG.5

INTERNATIONAL SEARCH REPORT

Internat al Application No
PCT/EP 99/01617

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07F9/572 A61K31/675 G01N33/58 C07F9/40 C07F9/6571

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07F A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 29691 A (GEORGIA TECH RESEARCH CORP) 9 November 1995 cited in the application see the whole document ---	1-34
Y	WO 95 34538 A (UNIVERSITAIRE INSTELLING ANTWERPEN) 21 December 1995 see the whole document ---	1-24
Y	FASTREZ J.: "Synthesis of new phosphonate inhibitors of serine proteases" TETRAHEDRON LETTERS., vol. 30, no. 49, 1989, pages 6861-6864, XP002105504 OXFORD GB see the whole document ---	1-34

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
17 June 1999	01/07/1999

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OLEKSYSZYN J ET AL: "IRREVERSIBLE INHIBITION OF SERINE PROTEASES BY PEPTIDE DERIVATIVES OF (A-AMINOALKYL)PHOSPHONATE DIPHENYL ESTERS" BIOCHEMISTRY, vol. 30, no. 2, 15 January 1991, pages 485-493, XP000168882 see the whole document	1-34
Y	ABUELYAMAN A S ET AL: "Synthesis and kinetic studies of diphenyl 1-(N-peptidylamino)alkane phosphonate esters and their biotinylated derivatives as inhibitors of serine proteases and probes for lymphocyte granzymes" ARCH. BIOCHEM. BIOPHYS. (ABBIA4,00039861);1997; VOL.344 (2); PP.271-280, XP002105505 The School of Chemistry and Biochemistry;Georgia Institute of Technology; Atlanta; 30332-0400; GA; USA (US) see the whole document	1-34
Y	LAMBEIR A M ET AL: "Dipeptide-derived diphenyl phosphonate esters: mechanism-based inhibitors of dipeptidyl peptidase IV" BIOCHIM. BIOPHYS. ACTA (BBACAQ,00063002);1996; VOL.1290 (1); PP.76-82, XP002105506 Laboratories of Medical Biochemistry and Pharmaceutical Chemistry, Department of Pharmaceutical Sciences, University of Antwerp (U.I.A.);Wilrijk; B-2610; Belg. (BE) see the whole document	1-34

2

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/EP 99/01617

Box I Observati ns where ertain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 32-34
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 32-34 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna' d Application No
PCT/EP 99/01617

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9529691 A	09-11-1995	US 5543396 A	06-08-1996
WO 9534538 A	21-12-1995	AU 2790895 A	05-01-1996
		EP 0764151 A	26-03-1997

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A61K 31/155, A61P 3/04, 3/10, 37/00

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FI99A000215	19 October 1999 (19.10.99)	IT

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(74) Agent: GERVASI, Gemma; Notarbartolo & Gervasi S.p.A., Corso di Porta Vittoria, 9, I–20122 Milan (IT).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF METFORMIN IN THE PREPARATION OF PHARMACEUTICAL COMPOSITIONS CAPABLE OF INHIBITING THE ENZYME DIPEPTIDYL PEPTIDASE IV

(57) Abstract

The use of metformin in the preparation of pharmaceutical compositions useful for inhibiting the enzyme dipeptidyl peptidase IV, in particular for increasing the plasma concentration of Glucagon–Like Peptide–1, is described.

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USE OF METFORMIN IN THE PREPARATION OF PHARMACEUTICAL COMPOSITIONS CAPABLE OF INHIBITING THE ENZYME DIPEPTIDYL PEPTIDASE IV

Field of invention

The present invention refers to the use of metformin in the preparation of pharmaceutical compositions useful for the treatment of pathologies requiring the inhibition of the enzyme dipeptidyl peptidase IV, in particular for increasing the plasma concentration of Glucagon-Like Peptide-1.

State of the art

It is known that the enzyme dipeptidyl peptidase IV (DPP-IV) is an enzyme present in the serum and expressed on the surface of endothelial cells in different parts of the body. This enzyme inhibits the action of Glucagon-Like peptide 1 (GLP-1), a hormone which stimulates insulin secretion and inhibits food intake, and of other hormones and neuropeptides including neuropeptide Y (NPY) and peptide YY (PYY). While the actions of the enzyme have not been completely characterised, it is known that DDP-IV is involved in the modulation of immune responses (it is known by immunologists as CD26).

Considering the wide spectrum of activity of DPP-IV, a product capable of inhibiting its activity could be very useful in the treatment of pathologies caused by a deficit (relative or absolute) of hormones degraded by the enzyme or by an excessive activity of CD26.

It is also known that Glucagon-Like Peptide-1 (GLP-1) is an hormone produced by the endocrine cells dispersed in gastrointestinal mucosae (Orskov et al. Endocrinology 119:1467-75 (1986)). This hormone is secreted mainly after meals

rich in carbohydrate (Shima et al. Acta Endocrinol Copenh 123:464-70 (1990)) and has two main effects:

a) it stimulates glucose-induced insulin secretion (Kreymann t al. Lancet 2:1300.1304 (1987)) and therefore is, at least partially, responsible for the increase of insulin secretion in the early post-prandial phase;

b) it inhibits food intake, through a direct action on the central nervous system (Turton et al. Nature 369:69-72 (1996)).

Since the hormone is capable of crossing the blood-brain barrier (Orskov et al. Diabetes 45:832-35, (1996)), the peripheral administration of the hormone determines a stimulation of central GLP-1 receptors involved in the regulation of food intake; therefore, it is conceivable that the gastroenteric post-prandial secretion of GLP-1 contributes to the induction of satiety after a meal.

The use of exogenous GLP-1 in therapy by parenteral (subcutaneous or intravenous) or trans-mucosal administration in type 2 diabetes has been widely investigated (Gutniak et al. Diabetes Care 20:1874-79 (1997), Rachman et al. Diabetologia 40:205-211 (1997)). The administration of exogenous GLP-1 during the meal is carried out in order to increase the secretion of insulin in the early post-prandial phase, correcting the insulin deficiency occurring in that phase in type 2 diabetes. An advantage of this therapy is that it also induces, facilitating body weight control. A product capable of increasing the post-prandial concentration of endogenous GPL-1 (increasing its secretion or reducing its inactivation) would therefore be useful in the treatment of type 2 diabetes and obesity. The inhibition of DPP-IV has been considered as a treatment option for type 2 diabetes, and

various studies are at present carried out in order to identify pharmaceuticals possessing such activity.

Type 2 diabetes mellitus is a disease characterised by a reduced sensitivity to insulin action (insulin resistance), associated with insufficient insulin secretion, particularly in the early post-prandial phase. In different patients one or the other pathogenic component can prevail: in general, in obese patients insulin resistance is considered the main pathogenetic mechanism, while in normal weight subjects with type 2 diabetes the deficit in insulin secretion is more evident and the insulin resistance less marked. These differences requires different therapeutic approaches: in obese patients drugs such as metformin, which increase insulin sensitivity, are preferred, while in normal weight molecules capable of stimulating insulin secretion (such as sulfonylureas) are more often used.

Metformin is an oral hypoglycemic of the biguanide class, widely used as a firts-approach therapy in overweight patients with type 2 diabetes.

The compound also shows a modest anorexic action; therefore, long-term metformin treatment usually reduced body weight, or prevents weight gain, in overweight type 2 diabetic patients.

Detailed description of the invention

It was now surprisingly found, and it is an object of the present application, that metformin, in the pharmaceutical form of administration usually employed and commercially available, inhibits the activity of DPP-IV and therefore can be useful for the preparation of pharmaceutical compositions to be used when the inhibition of such enzyme is requested.

In particular, and this is a second object of the present invention, it increases the plasma concentration of GLP-1, by stimulating hormone secretion and/or inhibiting its inactivation; metformin is therefore useful in the treatment of all the pathologic conditions where a deficit of GLP-1 is involved, without making it necessary the administration of exogenous GLP-1. More generally, considering the different effects of GLP-1, i.e. insulin secretion stimulation and satiety induction in the early post-prandial phase, the treatment with metformin can be useful in all pathologic conditions involving deficit of insulin secretion in the early post-prandial phase (for example type 2 diabetes, even in normal weight patients) and/or deficit of satiety (for example obesity, even when not associated with diabetes mellitus) and in any other pathology, at present not foreseeable, where the increase of plasma concentration of GPL-1 is required.

Experimental part

A) effect of metformin on GPL-1 secretion

The effect of metformine on the secretion of GPL-1 was studied on 20 obese non-diabetic male patients, aged 30-60 years, 10 of whom received metformin 850 mg per os t.i.d. for 14 days, while the remaining 10 patients received no treatment and were used as a control group. GPL-1 secretion is regulated by glycaemia and insulinaemia; since metformin reduces both glycaemia and insulinaemia, in order to verify the direct effect of the compound on hormone secretion and metabolism it was necessary to develop an experimental model wherein glycaemia and insulinaemia were maintained constant and controlled from the outside. The patients underwent an intravenous infusion of regular insulin (40 mU/m2*min) and glucose, and glucose infusion rates were adjusted on the basis of glycaemia in

samples of arterialised venous blood drawn every 5 minutes, in order to maintain glycaemia at 100 mg/dl (euglycemic hyperinsulinemic clamp), according to a technique described by DeFronzo et al. 1979. This procedure suppresses endogenous insulin secretion, and allows to maintain glycaemia and insulinaemia constant. After 90' from the beginning of the clamp, glucose (50 g) was administered orally, maintaining glycaemia constant by adjusting the glucose infusion rate accordingly. The circulating concentration of the active forms of GPL-1 (GLP-1[7-36]amide and GLP-1[7-37]) was measured at 0, 30, 60, and 90 minutes from the oral glucose load. This test was performed at the beginning of the study and after two weeks (at the end of metformin therapy for the active treatment group).

Metformin does not modify the basal concentration (i.e. those not stimulated by oral glucose administration) of GPL-1 (mean±SD after treatment in the active treatment group : 151±70 versus 132±56 pg/ml for GLP-1[7-36]amide, and 17±12 versus 19±15 for GLP-1[7-37]; p=NS at Student's paired t test). The treatment with metformin determined a relevant increase of GPL-1 levels after the oral glucose load: the incremental area under the curve (IAUC) increases from 2430±2781 to 10151±5058 pg*min/ml for GLP-1[7-36]amide and from 232±382 to 762±644 for GLP-1[7-37] ($p<0.05$ at Student's paired t test) in the active treatment group, while no significant variation is observed in the control group.

It is therefore clear, in the light of the above reported data, that orally administered metformin increases the plasma levels of the active forms of GPL-1 after an oral glucose load, without modifying the basal hormone concentration.

The here demonstrated action of metformin on an endocrine system (GPL-1) involved in the regulation of satiety can sugg st a wider use of th compound also on non-diabetic obese patient therefore beyond its present use (type 2 diabetes).

Moreover, since GPL-1 is a factor capable of stimulating insulin secretion it can be expected that metformin, through the stimulation of GLP-1, could have a stimulating effect on insulin secretion in the early post-prandial phase. This mechanism of action, ignored up to now, can be an hint to modify therapeutic treatment: metformin, in fact, should no more be considered as a molecule acting on insulin resistance only, and therefore especially suitable for obese diabetes patients, but it is a molecule with a peripheral effect (on insulin sensibility) and an effect, through GPL-1, on insulin secretion (stimulated in the early post-prandial phase).

B) Metformin inhibiting effect on the enzyme DPP-IV

To determine the inhibiting activity of metformine o DPP-IV its effect on the degradation of GLP-1(7-36) amide in vitro was studied by using a pool of plasma from voluntary human donors and in a buffer solution containing DPP-IV.

The plasma was collected from 11 healthy volunteers (6 men, 5 women) slim (body mass index <27 kg/m^2), with normal glucose tolerance, aged 25-42 years. The blood samples where collected at 8.30 in the morning, after overnight fast, in 10 ml ampoules containing EDTA and 500 UI of kallicrein; the plasma was immediately separated by centrifugation at 4°C. Samples of 1 ml of plasma were incubated for 30' at 37°C with 420 pg of GLP-1[7-36]amide in 0.1 M Tris HCl (pH 8) and with different concentrations (from 0 to 0.5 µg/ml) of metformin. The reaction was stopped after 30' by adding 1 ml trifluoroacetic acid 0.1% and the

samples were extracted on Sep-Pak C18 columns luted with acetonitrile in trifluoroacetic acid 0.1%. The eluates were lyophilised and stocked at –80°C.

420 pg/ml GPL-1[7-36]amide in Tris HCl 0.1 M 8pH8) were incubated with 0.06 U/ml of DPP-IV from pig kidney (Sigma, St. Louis. USA) in the presence of different concentrations (0 – 0.5 μg/ml) of metformin for 0 – 30' at 37°C. The samples were submitted to the same procedures described in the previous experiment.

The concentration of GPL-1(7-36)amide was measured (RIA) in the collected samples.

The concentration of GPL-1(7-36) amide measured in the samples at tempo 0, in the absence of metformin, was 356±21 pg/ml (theoretic 440 pg/ml) with a recover of 81%. The addition of metformin up to the highest concentration did not modify such concentration at time 0, showing that the metformin does not interfere with the laboratory determination of GLP-1[7-36]amide concentration. After an incubation of 30' at 37°C, GPL-1[7-36]amide concentration in serum decreases of 42% when compared to time 0. Metformin 0.1 and 0.5 μg/ml markedly inhibits such degradation in a dose-dependent manner; at 0.5 μg/ml metformin inhibits the degradation of GPL-1[7-36]amide almost totally. Similar results were obtained in the buffer solution containing DPP-IV.

The above reported data show that the inhibition of GLP-1 degradation caused by metformin is at least partly due to an inhibition of the activity of DPP-IV. That means that the action of metformin on the plasma concentration of GLP-1 is due, at least partially, to the inhibition of the hormone degradation by DPP-IV.

In this particular case the increase of the plasma concentrations of GPL-1 due to the inhibition of the DPP-IV activity can be useful in the treatment of different metabolic disorders as type 2 diabetes and obesity; in fat GLP-1 stimulates the secretion of insulin, reducing glycaemia, and at the same time it inhibits food intake inducing satiety. Moreover, as stated above, considering the different actions of this enzyme, the possibility of inhibiting its activity can be helpful for treating other pathologies where it can be useful to increase the concentrations of NPY, PYY or other possible hormones inactivated by DPP-IV. Moreover, the inhibition of the enzyme can prove useful in the treatment of pathologies of the immune system were the inhibition of CD26 activity is required.

As already said above metformin can be administered in the pharmaceutical forms commonly used and therefore in combination with the common excipients already used for the preparations of such forms, for example in the form of tablets.

The doses normally administered for the therapeutic treatment of the above said pathologies are comprised between 1000 and 2500 mg/die. A typical protocol of administration is for example a tablet containing 850 mg of metformine three times a day before breakfast, lunch and dinner.

CLAIMS

1. Use of metformine for the preparation of pharmaceutical composition useful to inhibit the enzyme dipeptidyl peptidase IV.

2. Use according to Claim 1 wherein the pharmaceutical compositions are useful to regulate the concentration of hormones and neuropeptides which are inactivated by the enzyme dipeptidyl peptidase IV.

3. Use according to Claim 1 wherein the pharmaceutical compositions are useful to regulate the immunity functions modulated by CD26.

4. Use according to Claims 2 wherein the hormone whose concentration is regulated is GLP-1.

5. Use according to Claim 2 wherein the neuropeptides whose concentration is regulated are the peptide YY and the neuropeptide Y.

6. Use according to claims 1 – 5 wherein the pharmaceutical composition consists of metformine and the usual carriers and excipients used for the preparation of oral forms.

7. Use according to Claim 6 wherein the pharmaceutical composition is in the form of tablets.

8. Method for inhibiting the activity of enzyme DPP-IV wherein a quantity of metformine comprised between 500 and 850 is administered to the patients 2-3 times a day.

9. Method for increasing the concentration of endogenous GLP-1 wherein a quantity of metformine comprised between 500 and 850 is administered to the patients 2-3 times a day.

10. Method according to claim 9 wherein the patient is an obese non diabetic subject.

11. Method according to Claim 9 wherein the patient is a diabetic slim or normo-weight subject.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/01849

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/155 A61P3/04 A61P3/10 A61P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, CHEM ABS Data, EMBASE, EPO-Internal, WPI Data, PAJ, BIOSIS, CANCERLIT, AIDSLINE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHEEN A.J. ET AL: "'About some non-conventional uses of metformin !. A PROPOS DE QUELQUES UTILISATIONS NON CONVENTIONNELLES DE LA METFORMINE." MEDECINE ET HYGIENE, (1992) 55/2173 (1492-1494). , XP000921039 figure 1 page 1492, column 2, paragraph 2 -page 1493, column 1, paragraph 4 --- -/--	1-11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2000	20/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cielen, E

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/01849

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 129, no. 17, 26 October 1998 (1998-10-26) Columbus, Ohio, US; abstract no. 211523, LUGARI, R. ET AL: "Effects of metformin on intestinal and pancreatic endocrine secretion in type 2 (non-insulin-dependent) diabetes" XP000920928 abstract the whole document & FRONT. DIABETES (1998), 14(MOLECULAR AND CELL BIOLOGY OF TYPE 2 DIABETES AND ITS COMPLICATIONS), 161-163 , ---	1-9,11
X	SCHEEN A.J.: "'How to treat... A non - obese patient with diabetes mellitus type 2!. COMMENT JE TRAITE... UN PATIENT DIABETIQUE DE TYPE 2 NON OBESE." REVUE MEDICALE DE LIEGE, (1994) 49/3 (121-122). , XP000921038 page 122, paragraph 2 - paragraph 3 ---	1-9,11
X	JACKSON R A ET AL: "Mechanism of metformin action in non-insulin-dependent diabetes" DIABETES, (1987 MAY) 36 (5) 632-40. , XP000921037 abstract table 1 --- -/--	1-9,11

2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/01849

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	PAOLISSO G (REPRINT) ET AL: "Effect of metformin on food intake in obese subjects" EUROPEAN JOURNAL OF CLINICAL INVESTIGATION, (JUN 1998) VOL. 28, NO. 6, PP 441-446. PUBLISHER: BLACKWELL SCIENCE LTD, P O BOX 88, OSNEY MEAD, OXFORD OX2 ONE, OXON, ENGLAND. ISSN: 0014-2972., vol. 28, no. 6, June 1998 (1998-06), pages 441-446, XP000218679 UNIV NAPLES 2, DEPT GERIATR MED & METAB DIS, SERV ASTANTERIA MED, PIAZZA MIRAGLIA 2, I-80138 NAPLES, ITALY (Reprint);UNIV NAPLES 2, INST ENDOCRINOL, I-80138 NAPLES, ITALY abstract page 441, column 1, paragraph 1 -column 2, paragraph 2 page 442, column 1, paragraph 3 page 445, column 1, paragraph 2 - paragraph 3 page 445, column 1, paragraph 5 -column 2, paragraph 2 page 446, column 1, paragraph 2 - paragraph 3 ---	1,2,5-10
X	ROURU J ET AL: "Anorectic effect of metformin in obese Zucker rats: lack of evidence for the involvement of neuropeptide Y." EUROPEAN JOURNAL OF PHARMACOLOGY, (1995 JAN 24) 273 (1-2) 99-106. , XP000920931 abstract figure 4 page 104, column 2, paragraph 3 - paragraph 4 page 105, column 1, paragraph 2 - paragraph 3 page 106, column 1, paragraph 2 ---	1,2,5,6
X	REYNOLDS J.: "Martindale - The Extra Pharmacopeia. Edition 31" 1997 , ROYAL PHARMACEUTICAL SOCIETY , LONDON, GB XP002141210 224540 page 357, column 2, paragraph 2 -column 3 --- -/--	8,9

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/01849

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 57634 A (SMITH STEPHEN ALISTAIR ;SMITHKLINE BEECHAM PLC (GB)) 23 December 1998 (1998-12-23) abstract page 1, line 1 - line 8 page 1, line 34 -page 2, line 25 page 4, line 10 - line 17 page 6, line 12 - line 32 claims 1,2,14,15,17,20,21 ---	1-9
P,X	STEFANOVIC V ET AL: "Reversal of increased lymphocyte PC-1 activity in patients with type 2 diabetes treated with metformin." DIABETES METAB RES REV, (1999 NOV-DEC) 15 (6) 400-4. , XP000921051 abstract page 401, column 1, paragraph 4 -column 2, paragraph 1 page 402, column 2, paragraph 4 table 4 page 404, column 1, paragraph 3 ---	1,8
P,X	DE 299 09 210 U (PROBIODRUG GES FUER ARZNEIMITT) 9 September 1999 (1999-09-09) page 1, paragraph 1 - paragraph 3 page 2, paragraph 1 - paragraph 2 page 4, paragraph 2 - paragraph 4 page 9, paragraph 3 -page 10, paragraph 2 page 11; table 1 ---	1,2,4,8, 9,11
A	TANAKA SUMIKO; MURAKAMI TAKANORI; HORIKAWA HIROSHI; SUGIURA MASAKI; KAWASHIMA KEISUKE; SUGITA TAKAHISA : "Suppression of arthritis by the inhibitors of dipeptidyl peptidase IV." INTERNATIONAL JOURNAL OF IMMUNOPHARMACOLOGY, vol. 19, no. 1, 1997, pages 15-24, XP000921050 abstract page 16, column 1, paragraph 1 - paragraph 2 page 20, column 1, paragraph 1 page 22, column 2, paragraph 2 - paragraph 3 ---	1,3
A	WO 98 19998 A (CIBA GEIGY AG ;VILLHAUER EDWIN BERNARD (US)) 14 May 1998 (1998-05-14) abstract page 1, paragraph 2 page 18, paragraph 3 page 19, paragraph 2 -page 20, paragraph 1 ---	1-11

2

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte...ational Application No
PCT/EP 00/01849

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOLST JENS J; DEACON CAROLYN F : "Inhibition of the activity of dipeptidyl-peptidase IV as a treatment for type 2 diabetes. " DIABETES , vol. 47, no. 11, November 1998 (1998-11), pages 1663-1670, XP000853619 abstract page 1663, column 2, paragraph 1 page 1664, column 2, paragraph 2 -page 1665, column 2, paragraph 2 page 1666, column 1, paragraph 5 -column 2, paragraph 2 page 1667, column 1, paragraph 2 -column 2, paragraph 1 page 1667, column 2, paragraph 3 page 1668, column 1, paragraph 2 -----	1-11

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 00 01849

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-8 relate to a use and method, defined by reference to the parameter "to inhibit the enzyme dipeptidyl peptidase IV". Moreover, claims 2,4-7 relate to the parameter "to regulate the concentration of hormones and neuropeptides which are inactivated by the enzyme dipetidyl peptidase IV", claim 3 relates to a use defined by reference to the parameter "to regulate the immunity functions modulated by CD26", claims 4,6,7,9-11 relate to the parameter "regulation of the concentration of GLP-1" and claims 5-7 relate to the parameter "regulation of the concentration of peptide YY and neuropeptide Y". Since the pharmacological action of this compound is not well-defined, the use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, the search has been restricted to the diseases specified in claims 10 and 11, namely obesity in non-diabetic subjects and diabetes in slim or normoweight subjects, with due regard to the general idea underlying the application.

Claims searched partially: 1-11.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.